

## INVITED REVIEW

# Visualization and functional characterization of lymphoid organ fibroblasts\*

Lucas Onder | Hung-Wei Cheng | Burkhard Ludewig 

Institute of Immunobiology, Medical Research Center, Kantonsspital St.Gallen, St.Gallen, Switzerland

**Correspondence**

Burkhard Ludewig, Institute of Immunobiology, Medical Research Center, Kantonsspital St.Gallen, St.Gallen, Switzerland.

Email: burkhard.ludewig@kssg.ch

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**Abstract**

Fibroblastic reticular cells (FRCs) are specialized stromal cells of lymphoid organs that generate the structural foundation of the tissue and actively interact with immune cells. Distinct FRC subsets position lymphocytes and myeloid cells in specialized niches where they present processed or native antigen and provide essential growth factors and cytokines for immune cell activation and differentiation. Niche-specific functions of FRC subpopulations have been defined using genetic targeting, high-dimensional transcriptomic analyses, and advanced imaging methods. Here, we review recent findings on FRC-immune cell interaction and the elaboration of FRC development and differentiation. We discuss how imaging approaches have not only shaped our understanding of FRC biology, but have critically advanced the niche concept of immune cell maintenance and control of immune reactivity.

**KEYWORDS**

cell-fate mapping, fibroblastic reticular cells, immune cell niches, lineage tracing, transgenic mouse models

## 1 | THE FIBROBLASTIC RETICULAR CELL NETWORK—A BRIEF HISTORY

Fibroblasts of lymphoid organs, also known as fibroblastic reticular cells (FRCs), build and maintain microenvironmental niches that host and nurture innate and adaptive immune cells.<sup>1,2</sup> FRCs generate the three-dimensional fiber and conduit channel networks that form the physical scaffold of secondary lymphoid organs (SLOs) and contribute to the fluid homeostasis of the tissue.<sup>3</sup> The regional expression of cytokines by FRCs guides the migration and interaction of different immune cell populations in SLOs.<sup>4</sup> Specialized FRC subsets provide growth factors to maintain distinct immune cell populations in dedicated niches and regulate immune cell interactions by on-demand supply of differentiation factors.<sup>5</sup> FRC subset definition and functional characterization have been fostered through the application of single cells transcriptomics analyses of FRCs in murine SLOs.<sup>6-11</sup> In broad

strokes, the conserved zonal segregation of SLOs is underpinned by four major FRC subsets: marginal reticular cells (MRCs) line epithelial or endothelial barriers and form antigen-sampling zones; B-cell zone reticular cell (BRC) networks generate environments for the generation of humoral immunity and include follicular dendritic cells (FDCs) that present native antigen to B cells; T-cell zone reticular cells (TRCs) define areas for T cell activation and differentiation and foster the interaction of T cells with dendritic cells (DCs); perivascular reticular cell (PRCs) are connected to blood vessels and possess the potential to act as embryonic and adult FRC progenitors. Despite their phenotypical and functional diversity, all FRC subsets show a conserved pattern of extracellular matrix (ECM) deposition and spindle-like morphology with the formation of dendritic protrusions and veil-like extensions, which are required to provide the fibrous scaffold and to generate liquid- and ECM-free spaces for direct membrane interactions with and between immune cells.<sup>3,12</sup> In this review, we focus on

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the immune-anatomy underlying FRC subset definition and the elaboration of functional properties through the combination of imaging and genetic FRC targeting. Since the field of stromal (FRC)-immune cell interaction has only recently gained substantial traction and the nomenclature still needs to be consolidated, a short overview on the history of key discoveries in FRC biology and the connection with the advancement of imaging techniques is warranted.

The first descriptions of “reticular cells” in B cell follicles of spleens and lymph nodes in rats have been published in the early 1950s. It had been noted that a network of channels and chords, termed the reticulo-endothelial system, exists in lymphoid organs. The reticulo-endothelial system could be highlighted by the deposition of intravenously injected proteins, suggesting that the involved cells had a function in the retention of antigens.<sup>13</sup> Such experimental approaches became feasible with the availability of fluorophores, such as fluorescein isothiocyanate, and the covalent coupling to antibodies directed against bovine albumin that enabled immunohistochemical detection of antigen deposition in lymphoid organs.<sup>13</sup> The recording of the antigen deposition in the reticulo-endothelial system was facilitated with the concomitant development of innovative sectioning techniques, such as dry ice-cooled microtomes for the production of native thin sections, and the first fluorescence microscopes. The initial interpretation drawn from the deposition of bacterial polysaccharides in subcapsular and follicular regions of murine lymph nodes was that the antigen is retained by macrophages and can transiently attach to lymphocytes.<sup>13,14</sup> More than 10 years later, tracing of *Salmonella adelaide*-derived flagellar antigens in rat lymph nodes revealed a cellular reticulum in follicles, which trapped the antigen on reticulin fibers.<sup>15</sup> Further studies using radioactive iodine-labeled *Salmonella*-derived flagellin as antigen revealed the connection between antigen retention on (“dendritic”) reticular cells in primary and secondary follicles/germinal centers that appear during B cell memory responses.<sup>16</sup> Additional seminal studies by Nossal and colleagues<sup>17</sup> revealed the discrete distribution patterns of radioactively labeled antigen on reticular networks in the center of primary follicles and more superficial (perifollicular) aspects of secondary follicles (Figure 1A). The authors speculated that the antigen-retaining reticular networks were generated by “dendritic macrophages,” an interpretation that was further promoted in studies using the detection of radioactive iodine-labeled flagellar antigen by transmission electron microscopy.<sup>18</sup> Antigen was identified near the surface of fine cell processes belonging to branches of “dendritic follicular reticular cells”. The authors also observed that bona fide “tingible body” macrophages of germinal centers did not participate in antigen retention.<sup>18</sup> The most commonly used term for antigen-retaining FRCs in the germinal center still follows the nomenclature established in the 1980s, that is, follicular dendritic cells (FDCs)<sup>19</sup> (Figure 1A,B). Later studies revealed that FDCs express surface proteins shared with other lymphoid organ fibroblasts,<sup>20</sup> that they are resistant to radiation and not replaced by bone marrow-derived cells,<sup>21</sup> and that they originate from perivascular myofibroblastic progenitors.<sup>22</sup> The initial (and still prominent) confusion of one of the key FRC subsets as a dendritic (myeloid) cell population is indicative for the eminent function of lymphoid organ fibroblasts in immunological processes.

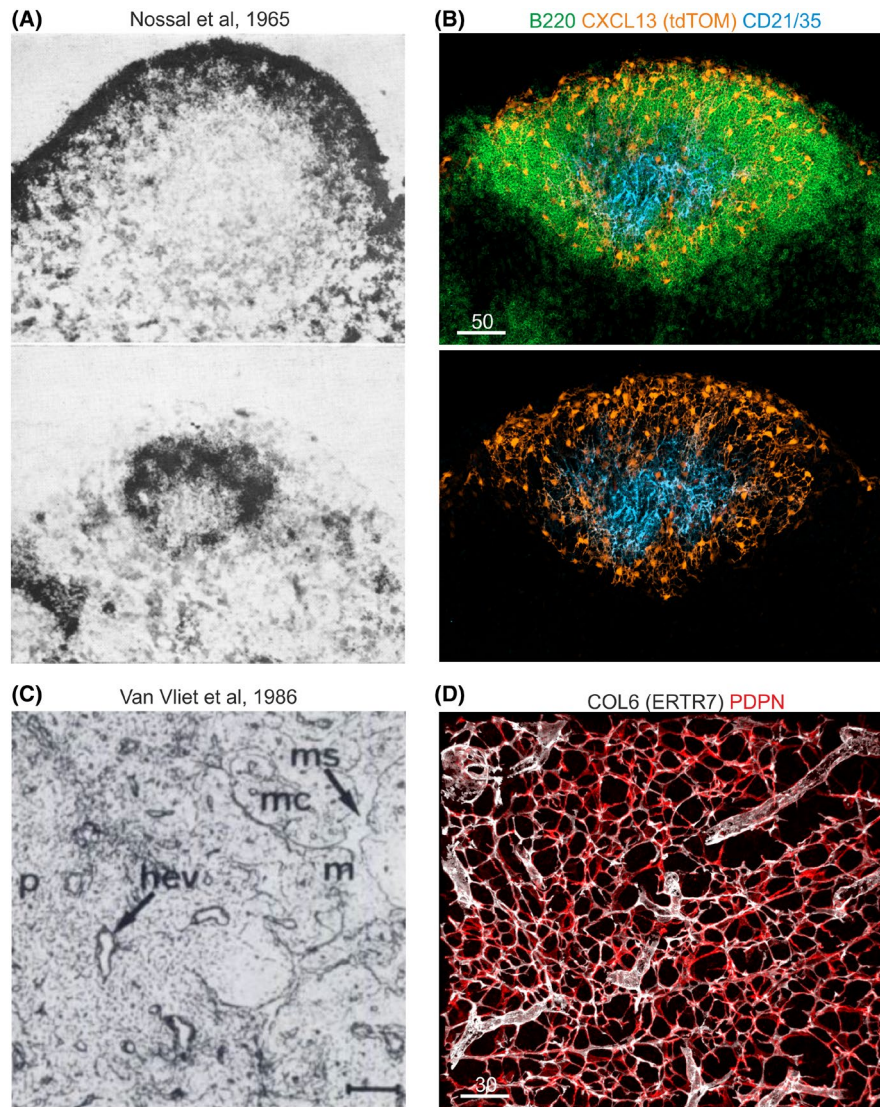
Research into the non-hematopoietic cellular components that contribute to the spatial organization of lymphoid organs and interact with immune cells increased during the 1970s and precipitated the generation of antibodies that recognize proteins expressed by lymphoid organ fibroblasts (Table 1). For example, the antibody Erasmus University Rotterdam-thymic reticulum antibody 7 (ER-TR7) enabled specific staining of reticular networks in the splenic white pulp and the T cell zone of lymph nodes<sup>23,24</sup> (Figure 1C,D). The ER-TR7 antibody recognizes collagen type VI<sup>25</sup> and facilitates hence the visualization of the extracellular matrix organization generated by the FRC network. Improved and refined immunohistochemistry protocols and the acquisition of high resolution images by laser scanning confocal microscopy and transmission electron microscopy revealed that the reticular fiber network, that is generated and ensheathed by FRCs, serves as a conduit for soluble factors and antigens.<sup>26-28</sup> FRCs have been further characterized through the expression of the chemokines CCL19 and CCL21<sup>29</sup> supporting the view that FRCs tightly interact with migrating CCR7-positive T cells and dendritic cells in the T cell zone. CXCL13 was identified as B cell attractant, secreted by fibroblasts underpinning lymph node follicles (ie, FDCs).<sup>30</sup> Intravital two-photon microscopy experiments revealed that T and B cells migrate on TRC and BRC networks of murine lymph nodes.<sup>21</sup> The importance of FRC-lymphocyte interaction was demonstrated by PDPN-positive FRC-mediated improvement of T cell survival in murine lymph nodes,<sup>31</sup> FRC-dependent coordination of lymph node remodeling following viral infection,<sup>32</sup> and the immune-regulatory role of FRC-derived nitric oxide.<sup>33,34</sup> The diverse functions of FRCs during immune homeostasis and activation indicate that such specialization depends on the generation of specialized microenvironmental niches for coordinated immune cell interaction and maintenance. The elaboration of FRC-generated immune niches was supported by the establishment of genetic tools to visualize and functionally manipulate FRC subsets in vivo.

## 2 | GENETIC TRACING OF LYMPHOID ORGAN FIBROBLASTS

The array of antibodies listed in Table 1 has enabled refined immunohistochemical definition of FRCs and has opened pathways for flow cytometric analyses and the selection of markers that permit genetic targeting of different FRC populations. The knowledge about FRC-specific protein expression has helped to identify a number of promoters that drive the expression of real-time reporters and/or facilitate Cre-mediated genetic recombination, thereby paving the way for studies on origin, phenotype, and function of FRCs.

### 2.1 | Visualization of FRCs in the tissue context

The elaboration of FRC biology requires the visualization of FRCs and interacting immune cells in situ. Microscopic analysis relies on the optimal preservation of tissue structure during sample preparation, which includes dissection of the native tissue,



**FIGURE 1** Fibroblastic reticular cell networks. (A) Nossal et al<sup>17</sup> have analyzed the retention of flagellar antigens labeled with radioactive iodine (<sup>125</sup>I) in primary and secondary follicles of rat lymph nodes. Scintillation counting and autoradiography highlighted a reticular cell network, which retains antigen early in perifollicular (upper image) and later in the center of the follicle (lower image). Reprinted with the kind permission from Immunology (John Wiley & Sons). (B) Cxcl13 promoter activity revealed by the Cxcl13-Cre/tdTomato model as fluorescent staining in B cell zone reticular cell networks that underpin subcapsular and perifollicular regions of mouse lymph nodes. Central follicles exhibit follicular dendritic cells (FDCs), which express the Cxcl13-tdTomato transgene and complement receptors 1 and 2 (CD21/35). (C) Staining of mouse lymph node fibroblasts by Van Vliet et al<sup>24</sup> using the ER-TR7 antibody that binds to the extracellular matrix component collagen type VI and stains the reticular fibroblast network in paracortical (p) and medullary (m) regions, surrounding medullary sinuses (ms) and within medullary chords (mc). Reprinted with the kind permission from The Journal of Histochemistry and Cytochemistry (SAGE Publications). (D) ER-TR7 staining of the murine lymph node T cell zone marks the fibrillar core of the microfluidic conduit network, which is produced by podoplanin (PDPN)-expressing T cell zone reticular cells

preservation, and fixation procedures as part of stringent workflows (Figure 2A). For optimal imaging of FRCs in different tissue contexts, the consecutive steps in the overall process should be optimized. The best-suited tissue fixation strategy, that is, chemical or physical fixation, depends on the particular antigen-antibody interaction because different fixatives alter the antibody-binding site. Nevertheless, the use of chemical fixation with protein-crosslinking agents such as paraformaldehyde or glutaraldehyde has become the standard for the 3-dimensional assessment of FRC networks (Figure 2B,C). High-purity paraformaldehyde generally

preserves SLO tissue size, structure, and antigen-binding sites for the majority of FRC markers (Table 1), although concentrations between 1% and 4% (w/v), incubation temperature and time have to be optimized empirically for different staining protocols. In addition, paraformaldehyde-based fixation procedures allow for the production of thick tissue sections using standard sectioning procedures such as the vibratome. Agarose embedding of paraformaldehyde-fixed tissues at room temperature followed by vibratome-sectioning preserve tissue integrity and thereby improve the resolution of intracellular structures and FRC-immune

**TABLE 1** Markers, cellular targets, and basic methodological parameters for the imaging-based characterization of murine lymphoid organ fibroblasts

Marker <sup>a</sup>	Organs	FRC subset <sup>b</sup>	Methodological details <sup>c</sup>			References
			Sectioning	Fixation	Detection	
ACTA2	LN, SWP, PP	All, but FDC	C, V	PFA	IF	9,11,31,52
CCL21 / <i>Ccl21a</i>	LN, SWP, PP	TRC, TBRC	C, V	PFA	IF, IHC	9,11,117,118
	LN		C, V	PFA	RNA	88,104
CXCL13 / <i>Cxcl13</i>	LN	BRC, MedRC	V	PFA	IF	8,119
	LN		C	PFA	RNA	88
	SWP, PP	BRC	V	PFA, Ace	IF	9,120,121
CD34	LN	MedRC	C	PFA	IF	6
	PP	TRC1	V	PFA	IF	11
CD35 (CR1)	LN, SWP, PP	FDC, LZ-FDC	C, V	PFA, Ace	IF, IHC	11,22,122
CD157	LN, SWP, PP	All, but MedRC, PRC	C	Ace	IF	123
<i>Ch25h</i>	LN	IFRC, TBRC	C	PFA/EtOH	RNA	6,100
COL1	LN, PP	All, but FDC	C, V	PFA	IF	11,88
COL6	LN, PP	All, but BRC	C, V	PFA	IF	21,52,124
	SWP	TRC, MRC	C	PFA	IF	125,126
DESMIN	LN, SWP, PP	All subsets	C, V	PFA	IF	21,52,127
FcεR2A	LN	LZ-FDC	V	PFA	IF	8
ICAM1	LN, SWP, PP	All, but PRC	C, V	Ace	IF	123
<i>Inmt</i>	LN	MedRC	C	PFA	RNA	6
MADCAM1	LN, SWP	MRC	V	PFA	IF	8,9
	PP	MRC, FDC, TBRC	V	PFA	IF	11
MYH11	LN	LZ-FDC	V	PFA	IF	8
LAMININ	SWP, PP	All subsets	C	PFA	IF	124,128
LEPR	LN	All subsets	C	PFA	IF	118
LUMICAN	LN	MedRC	V	PFA	IF	8
	PP	TRC, TBRC	V	PFA	IF	11
PDGFRβ	LN	All, but BRC	C	PFA	IF	129
PDLIM3	LN	DZ-FDC	V	PFA	IF	8
PDPN	LN, PP	All, but PRC	C, V	PFA	IF	52
	SWP	TRC	V	PFA	IF	9
<i>Pthlh</i>	LN	FDC	C	PFA	RNA	6
TNFSF11 (RANKL)	LN	MRC, IFRC	C, V	PFA	IF	6,8
	SWP, PP	MRC	C, V	PFA, AF	IF	11,130,131
TMEM119	LN	FDC	C	PFA	IF	6
VCAM1	LN, SWP, PP	All, but PRC	C, V	PFA, Ace	IF	120,123

<sup>a</sup>Murine lymphoid organ fibroblasts can be detected in situ using either protein or mRNA expression.

<sup>b</sup>FRC subsets in murine lymphoid organs include marginal zone reticular cells (MRC), interfollicular reticular cells (IFRC), light zone follicular dendritic cells (LZ-FDC), dark zone follicular dendritic cells (DZ-FDC), T-B border reticular cells (TBRC), B cell zone reticular cells (BRC) comprising MRCs, FDCs, TBRCs and IFRCs, T cell zone reticular cells (TRC), perivascular reticular cells (PRC), and medullary reticular cells (MedRC).

<sup>c</sup>Methodological details for the imaging of the respective FRC subset(s) in the indicated tissues according to the listed references include sectioning (cryo-sectioning (C) or vibratome-sectioning (V)), fixation (paraformaldehyde (PFA), paraformaldehyde and post-fixation with ethanol (PFA/EtOH), acetone fixation (Ace), or Antigenfix (AF)), and the detection method (immunofluorescence (IF), immunohistochemistry (IHC), RNAscope in situ hybridization with nucleotide probes (RNA)).

cell interactions (Figure 2D,E). The visualization and quantitative examination of 3-dimensional FRC network topologies require the definition of imaging volumes before sectioning. The approximate size of a single murine FRC ranges between 10 and 20 μm,

depending on the FRC subtype. Hence, the topological examination of FRC networks, for example in the T cell zone, requires at least 30 μm thick sections. Since human cells are bigger than their murine counterparts, the analysis of human lymphoid organ

fibroblasts generally requires the acquisition of even larger volumes. Future studies will most likely focus on the 3-dimensional positioning of multiple FRC communities to resolve the interconnection between different FRC subsets, comparable to the analysis of neuronal networks in the central nervous system.<sup>35</sup> Thus, an important step toward the goal of high-resolution analyses of extended FRC networks in murine and human SLOs is the acquisition of the whole organ (Figure 2F). Different tissue clearing procedures have been developed to minimize light absorption and scattering within the tissue in order to make large tissue volumes accessible for optical imaging.<sup>36</sup> Most of these protocols, such as 3DISCO and BABB, include organic solvent-based clearing and achieve optical clearance of murine organs.<sup>37</sup> Intact murine lymph nodes have been imaged and analyzed at single-cell resolution by the clearing-enhanced 3D-imaging ( $C_e3D$ ) pipeline,<sup>38</sup> which facilitates mainly antibody-based labeling of hematopoietic cells. In a similar approach, B cell dynamics during germinal center reactions<sup>39</sup> and clonally expanding FRC networks in developing and intact Peyer's patches<sup>11</sup> could be recently imaged at a larger scale using aqueous clearing agents.

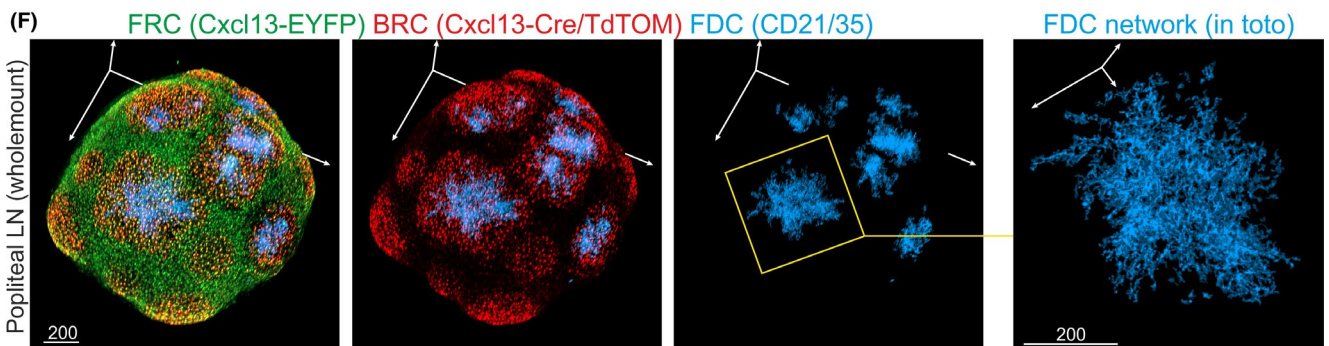
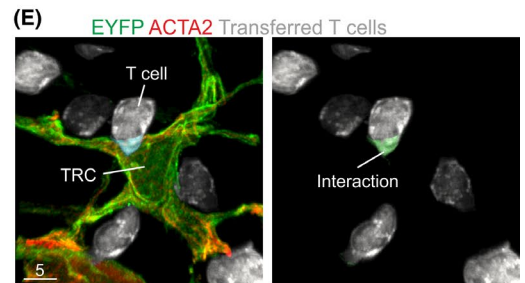
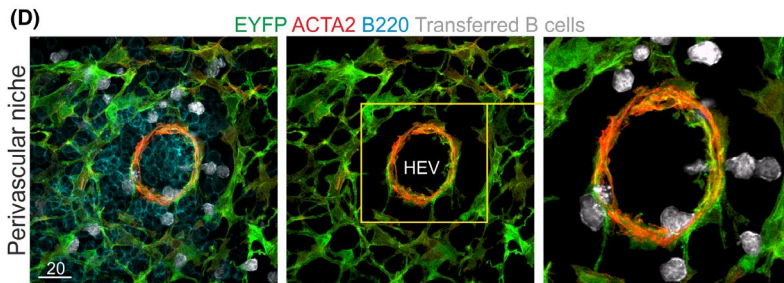
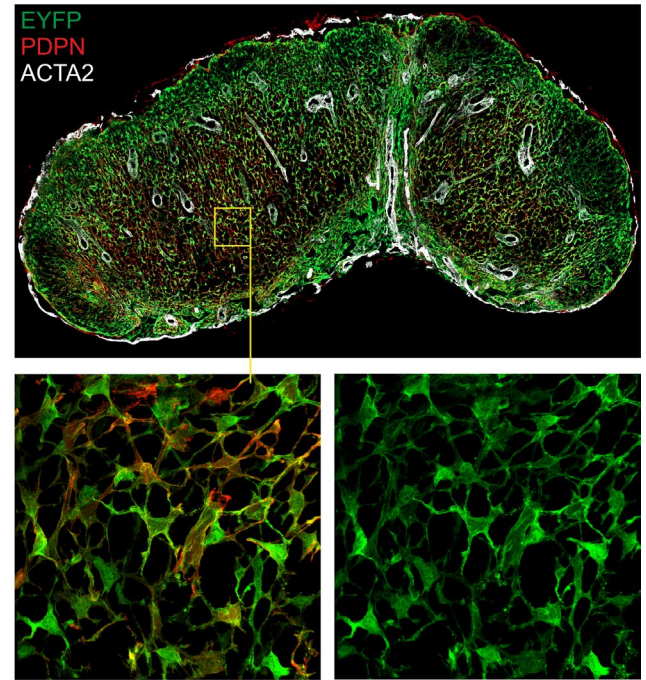
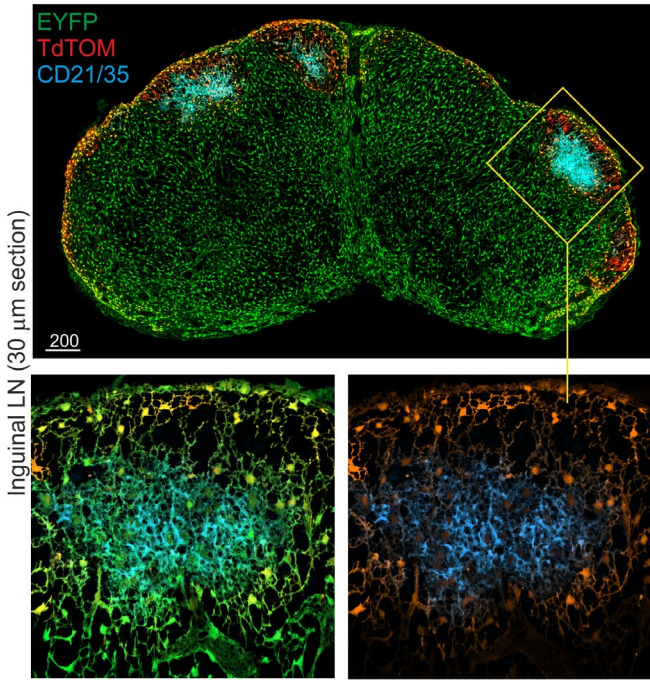
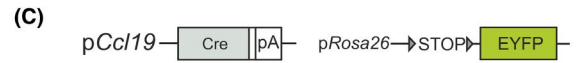
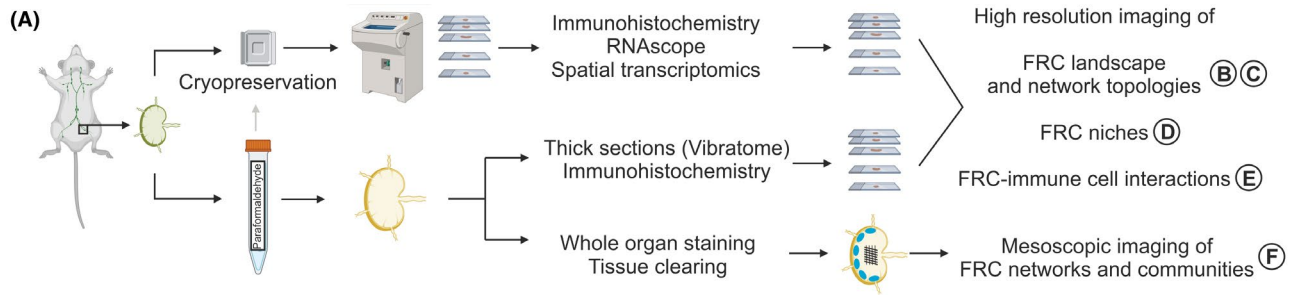
The continued improvement of confocal laser scanning microscopy (CLSM) has advanced the analysis of the 3-dimensional structure of the FRC landscape at high resolution. In particular, topological properties of the FRC and conduit networks<sup>12,40</sup> have been elaborated using CLSM. The 3-dimensional analysis of the TRC landscape at high resolution has resolved the small-world property of FRC networks that determines immune cell migration and lymph node functionality.<sup>12,40</sup> The formation of chemokine gradients along FRC networks guides the migration of immune cells, as shown for CXCL13 gradients present on the BRC network<sup>41</sup> or CCL21 gradients between the cortical and paracortical regions in murine lymph nodes.<sup>42</sup> Three-dimensional stromal cell network analysis by CLSM in combination with intravital two-photon microscopy revealed the territorial migration of lymphocytes on FRC networks in greater detail.<sup>21,40,43</sup> More recently, mesoscopic imaging technologies have been developed together with the advent of optical clearing

methods. For example, selective plane illumination microscopy<sup>44</sup> can be used to study dendritic cell-T cell interactions in whole murine lymph nodes<sup>45,46</sup> or the development of FRC networks covering the subepithelial dome and germinal centers of murine Peyer's patches.<sup>11</sup> Further technical progress in CLSM, mesoscopic imaging, and high-dimensional data analyses will facilitate the visualization and quantitative description of FRC network topology on a larger scale and will, at the same time, provide valuable information on cellular interactions and subcellular processes.

## 2.2 | Assessing FRC functions in genetic mouse models

Genetic targeting of different cell types and elaboration of their phenotype and function can be achieved through cell type-specific expression of the Cre recombinase and/or reporter genes. Cre recombinase expression cleaves genetically engineered Cre-recognition (lox-p) sites that flank transcriptional stop-cassettes upstream of single color reporter genes (eg, R26R-EYFP)<sup>47</sup> or stochastic multicolor reporter genes (eg, Brainbow transgenes)<sup>48</sup> to achieve permanent marker expression in the cells and their progeny. Moreover, the approach can generate conditional gain-of-function gene expression or conditional gene deficiency. A number of promoter constructs have been developed to drive Cre recombinase expression specifically in lymphoid organ fibroblasts (reviewed in<sup>5</sup>). The finding that murine lymph node FRCs can be highlighted by flow cytometry through a combination of PDPN and the endothelial cell marker CD31<sup>31</sup> had motivated the generation of a bacterial artificial chromosome (BAC) transgenic mouse line with Cre recombinase expression driven by the murine *Pdpr* promoter (*Pdpr*-Cre mice).<sup>49</sup> Cre recombinase-mediated reporter gene expression in *Pdpr*-Cre R26R-EYFP mice showed that only a small fraction of FRCs and lymphatic endothelial cells is targeted by this transgene.<sup>49</sup> Likewise, *Il7*-Cre mice<sup>50</sup> showed rather low transgene penetrance in murine lymph node FRCs<sup>51</sup> rendering these strains not suitable

**FIGURE 2** Multiscale imaging analyses of fibroblastic reticular cells (FRCs) in transgenic mouse models. (A) Dedicated workflows for lymphoid tissue preparation involve chemical and/or physical fixation strategies, which depend on the experimental question and the analyzed antigen combinations. The production of thin sections for immunohistochemical approaches or RNAscope analyses requires cryopreservation freezing of the tissue with subsequent sectioning using microtome cryostats. The assessment of the FRC landscape and network topologies can be achieved by producing thick sections from paraformaldehyde (or glutaraldehyde) fixed tissue, while global FRC networks and communities can only be imaged using stained and cleared whole-mount tissues. (B) Analysis of FRC landscapes using thick vibratome sections produced from *Cxcl13*-Cre/*tdTomato* R26R-EYFP lymph nodes (upper image) shows the presence of *tdTomato* expressing B cell zone reticular networks in the cortex of the lymph node. Confocal microscopy imaging of 30  $\mu$ m (or more) z-stacks facilitates the analysis of BRC networks underpinning B cell follicles (boxed area and images below). (C) Analysis of FRC landscapes using thick vibratome sections produced from *Ccl19*-Cre R26R-EYFP lymph nodes (upper image) show the presence of EYFP expressing FRC networks in the T cell zone and other niches of the lymph node. Confocal microscopy imaging of 30  $\mu$ m (or more) z-stacks facilitates the analysis of coherent TRC networks within the paracortex (boxed area and images below). (D) Perivascular reticular cells situated around high endothelial venules (HEV) and interacting with migrating B cells (transferred 4 hours prior to analysis) in lymph nodes of *Ccl19*-Cre R26R-EYFP mice were imaged at high resolution by Airy-scan microscopy. (E) TRC-T cell interactions were imaged on thick vibratome sections of *Ccl19*-Cre R26R-EYFP lymph nodes (T cells were transferred 4 hours prior analysis) at high resolution by Airy-scan microscopy. (F) *Cxcl13*-Cre/*tdTomato* R26R-EYFP lymph node stained in whole mount and cleared before imaging. The size of popliteal lymph nodes allows for adequate analysis of FRC networks in toto by mesoscopic imaging techniques such as selective plane illumination microscopy or conventional confocal microscopy



for studying the impact of conditional gene deletion in FRCs. It appears that the murine *Ccl19* promoter used in BAC transgenic mice to drive Cre recombinase expression is well-suited to functionally target lymphoid organ fibroblasts<sup>9,52</sup> (Figure 2C). The robust expression of Cre recombinase in *Ccl19*-Cre mice along the differentiation trajectory of FRCs from their embryonic progenitors to all adult progeny has facilitated the visualization and molecular characterization FRC development in lymph nodes,<sup>52</sup> spleen,<sup>9,53</sup> and Peyer's patches.<sup>11</sup> Moreover, functions of lymphoid organ fibroblasts during viral infection<sup>10,54,55,56</sup> or inflammatory diseases<sup>57</sup> have been elaborated. *Ccl19*-Cre activity can also be detected in FRCs underpinning non-classical SLOs such a fat-associated lymphoid clusters in the omentum (milky spots)<sup>58</sup> or tertiary lymphoid structures of the lung (ie, bronchus-associated lymphoid tissues).<sup>59</sup> The possibility to genetically target FRCs, as demonstrated for the *Ccl19*-Cre model, has advanced functional and phenotypical analyses of lymphoid organ FRCs and has paved the way to characterize FRC-like cells that appear as immune-interacting fibroblasts in inflamed tissues<sup>60,61</sup> and in the tumor microenvironment.<sup>62</sup>

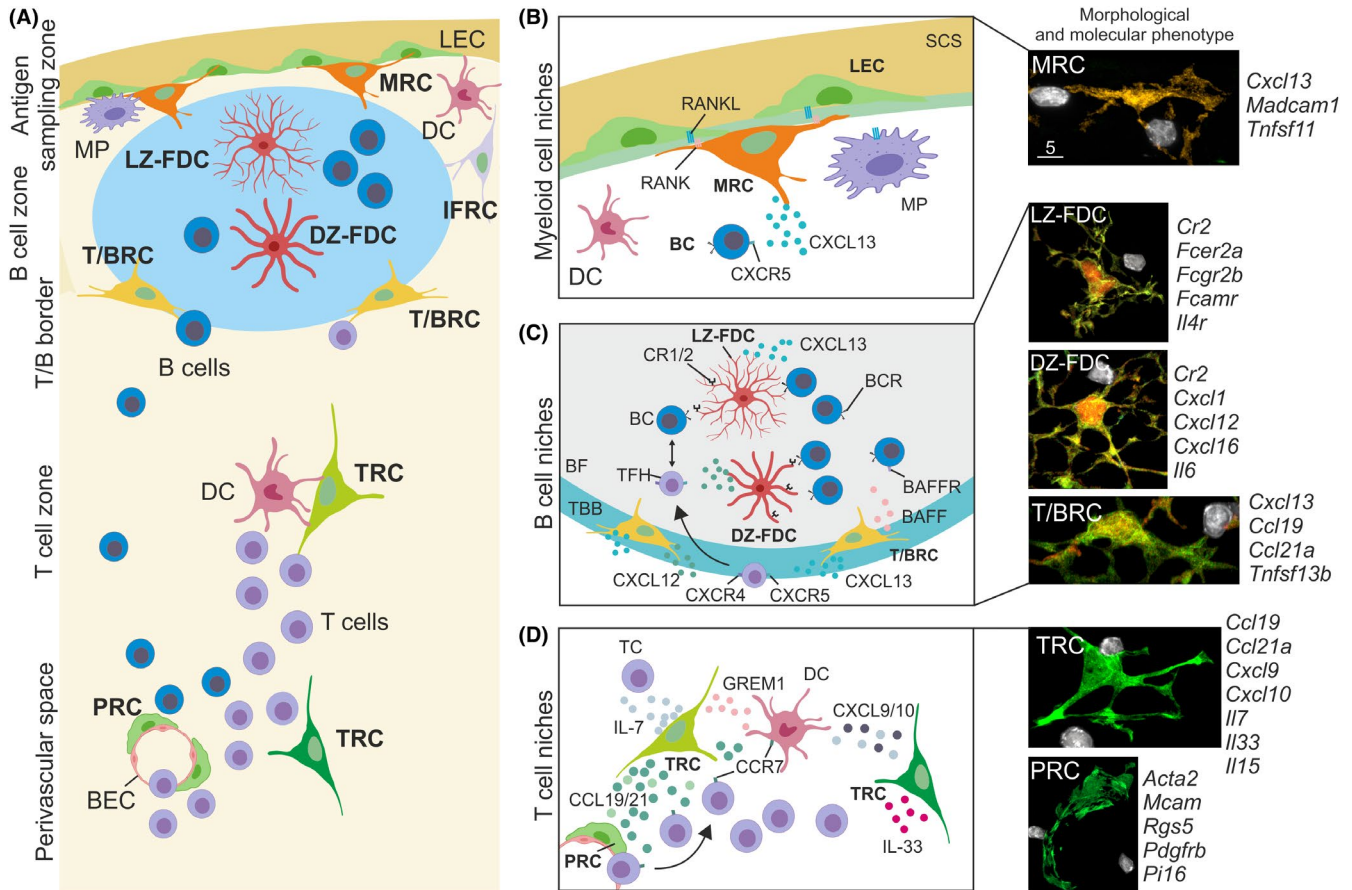
*Ccl19*-Cre transgene activity is initialized in all embryonic SLO anlagen during the third trimester of gestation and serves as a reliable lineage tracer for adult FRCs.<sup>9,11,63</sup> However, to elaborate the FRC progenitor differentiation pathways and to study the formation of specific FRC subsets, a FRC fate-mapping mouse model had to be developed. To this end, the BAC transgenesis strategy from *Ccl19*-Cre mice was applied to place the tetracycline transactivator (tTA) in combination with a tandem-repeated Tomato (tdTomato) reporter gene under the control of the *Ccl19* promoter (*Ccl19*-tTA/tdTomato).<sup>9</sup> *Ccl19*-tTA/tdTomato mice were crossed to LC-1 mice<sup>64</sup> to facilitate tetracyclin-mediated regulation of Cre recombinase expression. This inducible system was further crossed to the R26R-EYFP reporter (triple transgenic mice were named *Ccl19*-iEYFP) and enabled a timed control of Cre recombinase activity and imprinting of EYFP expression in FRC progenitors. High-resolution confocal microscopy of embryonic spleens in *Ccl19*-iEYFP mice identified periaarterial progenitors of splenic FRCs that give rise to all (known) FRC subsets of the splenic white pulp.<sup>9</sup> Differentiation trajectories of embryonic progenitors of Peyer's patch FRCs were studied in a combination of lineage-tracing and fate-mapping experiments using *Ccl19*-iEYFP mice.<sup>11</sup> Imaging of whole Peyer's patch anlagen of *Ccl19*-iEYFP mice during the perinatal period, when first immune cells populate the Peyer's patch primordia, identified perivascular progenitors that give rise to TRC and BRC subsets of adult Peyer's patches. Interestingly, early FRC progenitors clonally expand in the perivascular niche of the developing Peyer's patch, as demonstrated by imaging analysis of the fate of FRC progenitors in Peyer's patch primordia in *Ccl19*-iBrainbow mice.<sup>11</sup> A similar differentiation and clonal expansion was demonstrated for MRC and FDC subsets in B cell follicles of murine lymph nodes.<sup>65</sup> Taken together, deciphering progenitor-progeny relationships during FRC differentiation requires the combination of genetic fate-mapping tools with powerful imaging technologies.

Physical depletion of *Ccl19*-Cre-expressing cells has a fundamental impact on B cell follicle integrity and B cell survival.<sup>55</sup> However, since *Ccl19* mRNA expression is largely confined to TRCs and neighboring FRC subsets, certain fractions of BRCs are not targeted by this transgene, particularly in lymph nodes<sup>6,10</sup> and in Peyer's patches.<sup>11</sup> The notion that stromal expression of CXCL13 is restricted to B cell zones of adult SLOs<sup>30,66</sup> prompted the generation of *Cxcl13*-Cre/tdTomato dual reporter mice,<sup>63</sup> which facilitate lineage tracing by Cre-recombinase expression and detection of current *Cxcl13* promoter activity as tdTomato expression (Figure 2B). The dual reporter system facilitated assessment of virus-induced BRC remodeling and to dissect the molecular processes underlying FDC-mediated governance of the germinal center reaction.<sup>8</sup> The possibility to enrich CXCL13-expressing cells for single-cell transcriptomic analyses combined with CLSM-based in situ validation has led to the refined definition of B cell zone-underpinning BRC subsets including light zone (LZ)- and dark zone (DZ)-FDCs, MRCs, TBRCs, and interfollicular FRCs (IFRCs).<sup>8</sup> Furthermore, differentiation trajectory analyses of FRCs in *Cxcl13*-Cre/tdTomato mice confirmed the presence of CXCL13-expressing mesenchymal progenitors in early lymph node anlagen<sup>63</sup> that had been previously described by Mebius and colleagues.<sup>67</sup> Overall, the promoters of the chemokines CXCL13 and *CCL19* appear to be particularly well suited to genetically target lymphoid organ FRCs. This approach facilitates the delineation of functional properties through genetic ablation of critical pathways and the definition of different immune cell niches that are generated by specialized FRC subsets.

In sum, the continued progress in imaging approaches has defined FRC-generated immune cell niches as spatially confined microenvironments in lymphoid tissues that undergo remodeling during the course of immune responses. Furthermore, the detection of immunologically relevant molecules expressed by FRCs and the definition of molecular interfaces during the interaction with immune cells using high-resolution imaging (Figure 2E) provide important correlative information on FRC functions during immunological processes. The confirmation of the functional importance of particular immune pathways steered by FRCs within microenvironmental niches can be obtained through genetic manipulating of the FRC-immune cell crosstalk.

### 3 | FRC NICHES IN SECONDARY LYMPHOID ORGANS

The maintenance of immune cells and their differentiation within tissues requires the establishment of a niche environment that facilitates both long-lasting residence of a progenitor population and the regulated generation and differentiation of progeny. The niche concept has been developed by Schofield to explain the need for an environment that maintains "immortal" hematopoietic stem cells in the bone marrow.<sup>68</sup> The concept has been further extended to the presence of dedicated niches within tissues that support general



**FIGURE 3** Fibroblastic reticular cell niches in murine lymph nodes. (A) Lymph node compartments are underpinned by different fibroblastic reticular cell subsets, such as marginal zone reticular cells (MRC) situated below the subcapsular sinus in close contact with lymphatic endothelial cells (LEC) and macrophages (MP). B cell follicles harbor light zone follicular dendritic cells (LZ-FDC) and dark zone FDC (DZ-FDC) forming germinal centers and the adjacent B cell follicle areas are populated by interfollicular fibroblastic reticular cells (IFRC) and T-B border reticular cells (T/BRC). T cell zone reticular cells (TRC) are situated in the T cell zone and close to perivascular regions, tightly interacting with T cells and dendritic cells (DC). Blood vessels of lymph nodes are constructed by blood endothelial cells (BEC) and surrounded by perivascular reticular cells (PRC) forming the perivascular space, which functions as an area of immune cell trafficking from blood vessels into the lymph node parenchyma. (B) The myeloid cell niche is found in the antigen sampling zone of lymph nodes and is characterized by a multicellular crosstalk between MRCs, LECs, and MPs. MRCs are situated near the B cell follicle and closely interact with B cells, as shown in the right high-resolution micrograph with an MRC (orange) interacting with B cells (white). (C) B cell follicles harbor distinct B cell niches, such as the T-B border (TBB) containing TBRCs expressing chemoattractants to guide T follicular helper (TFH) and B cells to the B cell follicle. Central B cell follicles (BF) contain LZ- and DZ-FDC subsets, which present native antigen and instruct B cell differentiation in germinal centers. High-resolution images on the right show morphological features of BRC subsets (green, red) forming B cell niches and interacting with single B cells (white). (D) The T cell zone provides several niches for the activation and differentiation of T cells by the provision of cytokines and chemokines. Blood vessels traverse through the T cell zone and PRCs are involved in the recruitment of immune cells from the blood circulation. High-resolution images on the right show the TRC and PRCs morphology (green) of forming T cell and perivascular niches, respectively, and during the interaction with T cells (white). The adjacent molecular marker genes for FRC subsets have been derived from single-cell RNA-sequencing studies mentioned in the text. This figure was created using elements from BioRender.com

tissue maintenance,<sup>69</sup> the evolution of cancer stem cells,<sup>70</sup> and the provision of niche environments for specific immune cell populations such as macrophages.<sup>71</sup> Recent evidence from single-cell transcriptomics analyses has confirmed that fibroblastic stromal cells in perivascular spaces exhibit stemness/progenitor potential and support maintenance of different populations of lymphoid organ fibroblasts.<sup>72</sup> The FRC-dependent immune cell niche concept (Figure 3) includes the ability of lymphoid organ fibroblasts to receive and integrate signals from dynamic immune cell interactions and to provide

both maintenance and differentiation factors to different immune cells populations.

### 3.1 | The myeloid cell niche in antigen-sampling zones

FRC networks are highly heterogeneous in their topology depending on immune cell composition in the compartment that they



underpin (Figure 3A). Antigens and inflammatory components including pathogens, ligands of innate immune recognition receptors, chemokines, and cytokines, first encounter a layer of macrophages and dendritic cells in the antigen-sampling zone of the particular SLO. The antigen-sampling zone is underpinned by MRCs, which provide key signals for the sustenance and differentiation of myeloid cells. For example, lymph-borne antigens drain and accumulate in the subcapsular sinus of lymph nodes and are captured by subcapsular sinus macrophages<sup>73,74</sup> and dendritic cells of interfollicular regions.<sup>75</sup> The subcapsular sinus floor is built by lymphatic endothelial cells, which separate the lymphatic sinus from the cortex of the lymph node,<sup>76</sup> for example, B cell follicles and interfollicular regions. Lymphatic endothelial cells of the subcapsular sinus floor express atypical chemokine receptor 4 to scavenge CCL21 and to generate a CCL21 chemokine gradient that guides CCR7-expressing cells into the lymph node parenchyma during inflammation.<sup>42</sup> Lymphatic endothelial cells and MRCs form a niche that fosters the development of subcapsular sinus macrophages through the interaction between receptor activator of nuclear factor- $\kappa$ B (RANK, TNFRSF11A) and RANK-ligand (TNFSF11).<sup>77</sup> The dependence of subcapsular sinus floor lymphatic endothelial cells on the provision of RANK-ligand by MRCs<sup>77</sup> indicates that MRCs are part of a multicellular niche environment in the antigen-sampling zone of lymph nodes (Figure 3B). In addition, the subcapsular sinus floor macrophage niche is supported by lymphatic endothelial cell-mediated production of colony-stimulating factor 1 (CSF-1, macrophage colony-stimulating factor)<sup>78</sup> highlighting the importance of a multi-layered regulation within the niche environment.

In the splenic white pulp, blood-borne antigens are captured by marginal zone macrophages and specialized marginal zone B cells that reside in areas adjacent to the marginal sinus, which is formed by specialized blood endothelial cells. Splenic fibroblasts situated on both sides of the marginal sinus have been shown to support marginal zone B cell and red pulp macrophage survival.<sup>79,80</sup> Moreover, the sustenance and differentiation of marginal zone B cells require the expression of the Notch-2 ligand delta-like 1 by Ccl19-Cre-positive splenic FRCs.<sup>81</sup> Red pulp fibroblasts maintain red pulp macrophage populations through the production of CSF1 and replenish the red pulp myeloid cell compartment through the secretion of the chemokines CCL2 and CCL7,<sup>80</sup> indicating that the cellular dynamics within the antigen-sampling zone of the splenic white pulp is controlled by growth factors and the adaptive generation of migration and differentiation factors.

Peyer's patches are situated in the intestinal wall and have direct access to the content of the gut lumen. The antigen-sampling zone in Peyer's patches is located in a compartment known as the subepithelial dome, which is separated from the gut lumen by a single layer of follicle-associated epithelium. Antigens and inflammatory components from the gut lumen can traverse into the Peyer's patch parenchyma through specialized microfold epithelial cell (M cells) and are captured by myeloid cells for the presentation to B cells to promote IgA production.<sup>82</sup> Targeted ablation of RANK-ligand in Peyer's patch MRCs revealed that M cell differentiation depends on

functional MRC-M cell interaction.<sup>83</sup> A more recent study by Prados et al<sup>11</sup> showed that activation of Peyer's patch MRCs via the tumor necrosis factor receptor-1 is critical for M cell maintenance. In sum, the MRC niche in SLOs maintains and steers the activation of myeloid cells and forms critical interfaces with non-hematopoietic cells such as lymphatic endothelial cells in lymph nodes or epithelial cells in Peyer's patches.

### 3.2 | Dedicated B cell niches for immune cell positioning and activation

B cell follicles are situated directly adjacent to antigen-sampling zones in all SLOs and are underpinned by a network of CXCL13-expressing BRCs.<sup>6,8</sup> The BRC subset that forms the border between the T cell zone and B cell follicles (TBRCs) is a major source for the B cell survival factor BAFF.<sup>8,55</sup> Counter gradients of the chemokines CXCL12 and CXCL13 regulate B cell migration in primary B cell follicles and during the germinal center reaction.<sup>8,41,84</sup> The germinal center reaction triggers the reorganization of the B cell follicle into light zone (LZ) and dark zone (DZ) areas underpinned by phenotypically distinct FDC subsets.<sup>84,85</sup> Transcriptomic analysis of CXCL13-expressing BRCs further showed that LZ- and DZ-FDC subsets are present in the primary follicle and are poised to generate the respective microenvironments that steer the germinal center reaction through CXCL12-mediated positioning of follicular helper T cells and B cells<sup>8</sup> (Figure 3C). Furthermore, FDCs have been shown to promote the transition of LZ to DZ germinal center B cells through the activation of TGF $\beta$  signaling<sup>86</sup> and scavenge IL-4 at later stages of the germinal center reaction to foster the generation of memory B cells.<sup>87</sup> Interestingly, FRCs situated in the lymph node medulla (medullary reticular cells, MedRC) share transcriptomic similarities with BRCs.<sup>8,10</sup> It is most likely that the close interaction with plasma cells in the medullary chords stimulates MedRCs to provide IL-6 and BAFF to the interacting B cells.<sup>88</sup> It is noteworthy that while the BRC niche in murine lymph nodes has been studied extensively, the knowledge about the identity and activation of BRC subsets in Peyer's patches and the splenic white pulp is still limited. Clearly, the niche formed by BRCs is complex and conclusions concerning key niche factors need to take into account the proximity and partial overlap with the adjacent antigen-sampling zone and the T cell area.

### 3.3 | Dedicated niches control T cell activation and differentiation

Lymphoid organ fibroblasts occupying the T cell zone had been considered the major, or even only, FRC population, mainly due to the association of PDPN expression, IL-7 production and the effect of IL-7 on T cell homeostasis and T cell memory maintenance.<sup>31,89</sup> Recent single-cell transcriptomics analyses have indicated that the TRC landscape in murine SLOs is complex and consists of several TCR subsets.<sup>6,10,11</sup> The overarching function of TRCs is, in addition

to the formation of the reticular fiber and conduit network, the control of T cell and dendritic cell migration and their interaction<sup>21,40,90</sup> (Figure 3D). In addition, TRCs in Peyer's patches are essential to maintain the pool of type 1 innate lymphoid cells and conventional NK cells through the provision of the cytokine IL-15.<sup>54</sup> Parafollicular TBRCs expressing the bone morphogenic protein inhibitor GREM1 support the survival of conventional dendritic cells and/or their precursors.<sup>91</sup> Likewise, parafollicular FRCs express cholesterol-25-hydroxylase (CH25H) to produce hydroxycholesterol, a ligand for G protein-coupled receptor 183 (GPR183, also known as EBI2),<sup>6</sup> which is most likely a key step to direct the positioning of CD4<sup>+</sup> T cells in close contact with dendritic cells in parafollicular areas to foster T cell immunity.<sup>92</sup> It still remains unknown whether the expression of *Cxcl9* and *Cxcl10* by different TRC populations<sup>6,10,11</sup> warrants the assignment as a distinct subset. During viral infection, both dendritic cells and lymphoid organ fibroblasts acquire an activated cell status to coordinate the distribution and differentiation of effector and central memory T cells,<sup>93</sup> suggesting that T cell niches formed by TRC subsets can dynamically adapt during immune activation. Overall, specialized niches generated by different FRC subsets maintain immune cell homeostasis and facilitate dynamic and regional adaptation of particular microenvironments to secure immunocompetence during infection and the development of cancer.

## 4 | MOLECULAR MECHANISMS GOVERNING FRC-IMMUNE CELL INTERACTION

The FRC network in SLOs accommodates immune cells to facilitate their positioning, activation, and differentiation. Importantly, FRC niches rapidly adapt to changing immune cell dynamics during the course of immune responses. The visualization and quantitative assessment of these processes represent a substantial experimental challenge. The major approach to resolve the processes underpinning FRC-immune cell interactions over time has been—and still is—the sequential visualization, mainly by CLSM, of static snapshots during immune responses. In addition, real-time imaging by intravital two-photon microscopy of immune cell migration in SLOs has revealed the importance of the FRC network for the guidance and activation of lymphocytes. The perturbation of FRC-immune cell interaction by infection, cancer, or autoinflammatory triggers in combination with advanced imaging methods has provided key insight into the mechanisms underlying FRC-immune cell interaction.

### 4.1 | Immune cell migration is guided by FRCs

The compartmentalization of SLO structures is determined by FRC subsets that produce distinct sets of chemokines and generate gradients of cholesterol derivatives to orchestrate immune cell migration and positioning. Seminal work by the Germain group has shown by intravital two-photon microscopy that naive T and B cells enter

lymph nodes through high endothelial venules and migrate along the FRC network toward the T or B cell zones.<sup>21</sup> The entry of lymphocytes into lymph nodes via the high endothelial venule route depends mainly on the expression of CCR7 and the production of CCL19 and CCL21 by TRCs.<sup>29</sup> Moreover, assessment of the migration of lymph-derived T cells into the lymph node parenchyma by a combination of CLSM and intravital imaging revealed that T cells utilize CCR7-dependent routes through medullary sinuses, whereas dendritic cells transmigrate through the floor of the subcapsular sinus.<sup>94</sup> It remains to be established which additional FRC-derived chemokine signals are required for the establishment of cell type-specific intranodal migration. Similar challenges remain to be mastered for the full elucidation of T cell migratory pathways in the spleen, where lymphocytes gain passively access to the red pulp via arterioles that end in the sinuses of the red pulp. Real-time imaging in combination with genetically modified T cells and genetically highlighted PRCs and TRCs in the white pulp has revealed that naive T cells migrate from the red pulp into the white pulp via perivascular tracks.<sup>43</sup> CCR7-dependent T cell entry into the splenic white pulp occurs through FRC-underpinned bridging channels in the marginal zone.<sup>90</sup> T cells then follow a CCL19 chemokine gradient into the T cell zone that is formed by PRCs.<sup>43</sup> It appears that naive B cells follow the same migration route to transit from the red pulp into the white pulp.<sup>90</sup> However, it is still unclear which FRC-derived migratory cues, that is, chemokine gradients, guide B cells along the TRC scaffold into the B cell follicles.

Antigen-presenting cells such as dendritic cells secure surveillance of peripheral tissues through transport of immunologically relevant information, that is, pathogens, antigens, and inflammatory substances, to lymph nodes. Migratory dendritic cells enter lymph nodes via afferent lymphatics and transmigrate through the subcapsular sinus toward the T cell zone guided by CCL19/CCL21 gradients that are generated by TRCs.<sup>94</sup> Intravital two-photon microscopy has shown that the CCR7-dependent initial migration of activated dendritic cells from skin through lymphatic vessels to draining lymph nodes is fostered by immune complexes.<sup>95</sup> Immune activation, for example following influenza vaccination, also activates intranodal DC migration as revealed by live-imaging of T cell zone-resident DCs that move toward the medulla to capture antigen and to initiate the immune response.<sup>96</sup> Such inflammation-associated intranodal migration is most likely enhanced by the expression of the chemokines CXCL9 and CXCL10 by multiple immune cell populations including dendritic cells and T cells,<sup>96</sup> and by several FRC subsets.<sup>6,10</sup> DCs situated in medullary sinuses can directly capture lymph-borne antigens and migrate to the T-B border to steer activation of humoral immune responses.<sup>97</sup> Positioning of particular dendritic cell subsets at the T-B border is directed by the chemoattractant receptor EBI2/GPR183<sup>92</sup> that senses oxysterol concentration gradients and determines the positioning of immune cells in SLOs.<sup>98</sup> The enzyme CH25H that synthesizes oxysterol derivatives is expressed by MRCs, TBRCs, and IFRCs in the parafollicular areas,<sup>6</sup> whereas the oxysterol metabolizing enzyme HSD3B7 is enriched in stromal cells of the T cell zone.<sup>99</sup> Quantitative immunohistochemistry has shown

that the differential expression of these enzymes regulates oxygen gradients in the spleen and directs the migration of CD11b<sup>+</sup> conventional type 2 dendritic cells to the T-B border to stimulate B cell activation.<sup>100</sup> In sum, classical and advanced imaging approaches have been shown to be important to elaborate how the FRC network generates pathways for immune cell migration to and within SLOs and to characterize chemokine and cholesterol metabolite gradients that foster immune cell interaction.

## 4.2 | FRC-tailored microenvironments for immune cell activation and differentiation

Immune activation leads to fundamental remodeling processes in SLOs, mainly due to rapid changes in immune cell influx or efflux and shuffling of immune cells between different niche environments. The influx of antigen-presenting cells and other immune cells increases overall cell numbers and FRC networks physically adapt to these changing cell densities.<sup>3</sup> Analysis of lymph node swelling reactions by high-resolution CLSM deconvoluted the physical stretching behavior of the FRC cell body and the FRC network to compensate for the changing space restrictions during the early phase of a response when immune cell influx and proliferation increase.<sup>101</sup> Stretching of FRC networks requires the inhibition of PDPN-mediated contraction through interaction with C-type lectin receptor on dendritic cells.<sup>101,102</sup> Importantly, dendritic cells sustain FRC differentiation during this highly dynamic process through the induction of lymphotoxin beta receptor signaling and thereby foster FRC proliferation in swelling lymph nodes.<sup>103,104</sup> FRCs undergo profound phenotypical changes during immune activation including enhanced production of effector cytokines such as IL-4, IL-33, and IL-6,<sup>105,106</sup> and increased expression of surface molecules that modulate T cell activation such as inducible T cell costimulator ligand,<sup>106</sup> major histocompatibility complex molecules<sup>58</sup> or co-inhibitory molecules.<sup>10</sup> The fine-tuned expression of cytokines and other stimulatory factors by FRCs are decisive for the differentiation of T cells in effector or helper subsets and establish setpoints for ongoing immune response. Conversely, FRCs receive important signals from immune cells, for example through the type 1 interferon receptor alpha, which is necessary to control local lymphocytic choriomeningitis virus infection.<sup>10</sup> Interestingly, the lack of IFNAR signaling in FRCs led to altered LCMV infection patterns in the lymph node FRC network as revealed by imaging of viral nucleoproteins in lymph nodes of acutely infected mice,<sup>10</sup> indicating that sensing of innate immunological signals is important for the control of infectious agents. During later phases of an acute immune response, FRCs contribute to the contraction of lymphocyte populations through the activation of the COX2 pathway,<sup>107</sup> expression of programmed death ligand 1,<sup>10</sup> or through the restriction of IL-15 availability.<sup>54</sup> Noteworthy, the important role of lymphoid organ fibroblasts in the activation and regulation of T cell responses as outlined in these studies could only be elaborated through the combination of functional approaches

with advanced imaging methods that facilitate the localization of FRC-immune cell interaction.

The FRC niche concept predicts that the dynamic regulation of FRC "catering," that is, provision of migration, growth, and differentiation factors is key for the regulation of immune responsiveness. Hence, one major challenge for the assessment FRC-immune cell interaction is the quantification of effector molecules and the determination of their distribution patterns. The understanding of FRC niche furnishment is particularly important during a multicellular crosstalk, for example during T follicular helper cell-B cell interaction in the course of antiviral B cell response when the expression of the chemotactic receptor CXCR5 is changing rapidly.<sup>108</sup> Indeed, the expression of the CXCR5 ligand CXCL13 by BRCs forms both soluble and immobilized gradients express complex gradients of these chemokines.<sup>41</sup> Quantitative assessment of such gradients on FRC small-world networks became feasible through the combination of high-resolution microscopy and computer simulations leading to the conclusion that mainly immobilized CXCL13 gradients promote B cell trafficking.<sup>41</sup> High-resolution confocal imaging has been most helpful to disentangle how and to what extent CXCL12 expression by TBRCs and DZ-FDCs supports T follicular helper cell-B cell interactions in the germinal center.<sup>8</sup> Clearly, further advances in physical, chemical, and biological system will generate tools to quantify the molecular and cellular mechanisms underlying the processes in FRC-generated niches with high fidelity. Improved and more accurate data acquisition will be suitable for the analysis in mathematical and computational models of immune function as has been recently achieved for the analysis of single-cell transcriptomics or high-dimensional flow cytometry data.<sup>109</sup>

## 5 | CONCLUSIONS AND OUTLOOK

The advancement of imaging approaches has been key for the initial description and the subsequent characterization of specialized lymphoid organ fibroblasts. Antibody-based staining and visualization are still the main methodological approach to provide insight into FRC morphology and phenotype (Table 1). The localization and quantification of protein expression by FRCs and the morphological characterization of different FRC subsets in tissue sections by high-resolution imaging have become particularly important for the validation of gene expression profiles that can be generated by single-cell transcriptomics. Genetic targeting of murine FRCs with expression of fluorescent markers has further advanced our understanding of FRC biology, mainly through the combination with Cre-mediated gain-of-function or gene ablation approaches. However, the imaging of immune dynamics has not yet fully exploited the sophisticated methods for *in vivo* FRC visualization and functional manipulation offered by the available genetic models. In sum, imaging approaches have guided and constantly improve the elaboration of the specialized immune cell niches in SLOs that are generated by FRCs.

One of the main challenges for future studies that aim at the resolution of FRC-immune cell interaction is the 3-dimensional positioning of multiple FRC communities, the interconnection between different FRC subsets, and the changes in FRC phenotype and function that are associated with lymphoid organ remodeling during infection, inflammatory diseases or cancer. The size of human lymphoid organs represents a similar challenge for the imaging-based characterization of FRC-immune cell interaction during disease processes. First efforts have been made to isolate and characterize human FRCs from tonsils,<sup>110</sup> lymph nodes,<sup>111</sup> or spleen<sup>112</sup> revealing the expression of the chemokines CCL21 or CXCL13 in situ. The remodeling of lymphoid organ structure and the associated phenotypic changes in the human FRC landscape has been highlighted during HIV infection<sup>113</sup> or in patients with follicular lymphoma.<sup>114</sup> Recent single-cell transcriptomic analyses of human tissues have revealed the cellular complexity of fibroblasts in lymphoid organs.<sup>72</sup> However, FRC community structures and the interaction patterns with immune cells are still elusive and need to be resolved using high-resolution and large-scale imaging methods. While conventional CLSM facilitates high-resolution imaging to assess morphological and subcellular detail, larger volumes cannot be acquired. In contrast, mesoscopic imaging is able to scan more than 50 mm<sup>3</sup> tissue volumes in human sections,<sup>115,116</sup> but does not permit in situ assessment of molecular detail. Hence, the combination of confocal, high-resolution, and mesoscopic imaging will be required for the full characterization of the FRC landscape in human lymphoid organs. This combined approach needs to be supported by mathematical and bioinformatics-based integration to define critical processes in FRC-generated immune cell niches that could be therapeutically targeted in infection, cancer, or autoimmune diseases.

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## CONFLICT OF INTEREST

LO, H.-WC, and BL are co-founders and shareholders in Stromal Therapeutics AG, St. Gallen, Switzerland.

## ORCID

Burkhard Ludewig  <https://orcid.org/0000-0002-7685-573X>

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