

Chapter 1

Leukocyte-Stromal Interactions Within Lymph Nodes



Joshua D’Rozario, David Roberts, Muath Suliman,
Konstantin Knoblich, and Anne Fletcher

Abstract Lymph nodes play a crucial role in the formation and initiation of immune responses, allowing lymphocytes to efficiently scan for foreign antigens and serving as rendezvous points for leukocyte-antigen interactions. Here we describe the major stromal subsets found in lymph nodes, including fibroblastic reticular cells, lymphatic endothelial cells, blood endothelial cells, marginal reticular cells, follicular dendritic cells and other poorly defined subsets such as integrin alpha-7+ pericytes. We focus on biomedically relevant interactions with T cells, B cells and dendritic cells, describing pro-survival mechanisms of support for these cells, promotion of their migration and tolerance-inducing mechanisms that help keep the body free of autoimmune-mediated damage.

Keywords Stromal cells · Lymph nodes · Fibroblasts · FRCs · Lymphoid fibroblasts · Lymphatic endothelium · Endothelial cells · LECs · Stromal Immunology · Podoplanin · Non-haematopoietic

1.1 Introduction

Lymph nodes are the most prevalent secondary lymphoid organ (SLO), contained in the neck, armpits, lungs, abdomen, collarbone, knee and groin regions [1]. They range in size from a few millimetres to over 2 cm and enlarge significantly under certain conditions involving immune activation, such as infection or cancer [1, 2].

J. D’Rozario · K. Knoblich (✉) · A. Fletcher (✉)
Department of Biochemistry and Molecular Biology, Monash University,
Clayton, VIC, Australia

Institute of Immunology and Immunotherapy, University of Birmingham,
Birmingham B15 2TT, United Kingdom
e-mail: konstantin.knoblich@monash.edu; anne.l.fletcher@monash.edu

D. Roberts · M. Suliman
Institute of Immunology and Immunotherapy, University of Birmingham,
Birmingham B15 2TT, United Kingdom

Lymph nodes are structurally organised and contain a cortex, paracortex and medulla, which are separated into different regions to allow the movement of lymph through the organ [3]. The cortex is situated beneath the capsule and subcapsular sinus with B lymphocytes and follicular dendritic cells contained within follicles present in the cortical region [4]. The paracortex lies deeper within the lymph node structure with T lymphocytes homing to these regions to interact with antigen-presenting cells [4]. The medulla consists of B lymphocytes and macrophages dispersed within medullary cords which allow for the movement of lymph from the cortex into efferent lymphatic vessels [4]. This structure allows for antigen-bearing antigen-presenting cells (APCs) and lymphocytes to efficiently interact within the lymph node, enabling an appropriate immune response against an invading pathogen [5]. The microenvironment of the lymph node is crucial for immune function and consists of endothelial cells lining lymphatics and blood vessels and fibroblastic reticular cells which create the internal reticular structure of lymphoid organs [6].

Lymph, which may bear soluble antigen, enters the lymph node through afferent lymphatic vessels, where it empties into the subcapsular sinus and then traverses through the medullary sinuses surrounding the medullary cords to interact with B cells [4]. The lymph filters through the cortex where it exits via the efferent lymphatic vessels contained in the hilus [4]. Dendritic cells (DCs) actively migrate into the lymph node via afferent lymphatics [7]. Dendritic cells then migrate to paracortical T cell zone using stromal cells as a scaffold for migration [8]. B and T cells also use the stroma to migrate, entering lymph nodes from the bloodstream through specialised high endothelial venules [9]. Following entry, B cells move to the B cell follicles in the cortex, while T cells move to the paracortical T cell zone where they can begin scanning arriving APCs for their cognate antigen [8, 10, 11].

Structural components of the lymph node are now broadly appreciated as primary regulators of the adaptive immune response [10, 12–26]. These lymphatic structural components, termed lymph node stromal cells (LNSCs), comprise of non-haematopoietic cells that can be divided into functionally and phenotypically distinct subsets based on surface expression of glycoproteins CD31 and podoplanin (gp38) with an absence of haematopoietic marker CD45 [14]. These include blood endothelial cells (BECs), lymphatic endothelial cells (LECs), integrin $\alpha 7+$ pericytes (IAPs), follicular dendritic cells (FDCs) and fibroblastic reticular cells (FRCs) [14, 22, 27]. These stromal cells play a variety of roles in lymph node homeostasis and function, as they interact with lymphocytes to create an optimal microenvironment for cell activation and migration.

1.2 Fibroblastic Reticular Cells (FRCs)

Selectable markers: Gp38+, CD31-, ER-TR7+, LT β R+, desmin+, aSMA+

FRCs are myofibroblasts that have evolved to create a specialised microenvironment within lymph nodes. FRCs are heterogeneous and exist in different niches within the lymph node, fulfilling unique immunoregulatory roles [27] (Table 1.1, Fig. 1.1a–f).

Table 1.1 Lymph node FRC subsets

FRC subtype	Characteristics	Phenotype	Function	References
T cell zone reticular cells (TRCs)	Secretion of CCL19, CCL21 and IL-7 within paracortex	PDPN+, desmin+, MAdCAM-, CCL19+, CCL21+	Maintaining T cell homeostasis Forming conduit network Allowing lymphocytes to migrate and interact efficiently on the 3D meshwork	[8, 11, 14, 22, 30, 33, 36]
B cell zone reticular cells (BRCs)	Located in (resident) or near (inducible) B cell follicles. They secrete BAFF and are induced during inflammation to produce CXCL13	Resident cells: PDPN+, CCL19+, BAFF+ Inducible cells: PDPN+, CXCL13+	Supporting B cell survival and follicle boundary integrity	[24, 25, 37, 38]
Marginal reticular cells (MRCs)	Located in subcapsular region Not found in tertiary lymphoid organs	PDPN+, desmin, MAdCAM1, IL-7 ^{hi} , CXCL13+, RANKL ^{hi}	Produce very high levels of IL-7. Precursor cell type for FDCs within lymph nodes	[39–41]
Follicular dendritic cells (FDCs)	Within lymph nodes, FDCs develop from MRCs but are nonetheless highly distinct from other FRC types. Located within primary and secondary B cell follicles. Secretion of CXCL13	CD21+, CD35+, MFGE8+, CXCL13+, ICAM1+, VCAM1, BAFF+	Maintains germinal centre integrity. Facilitates the production of high-affinity antibodies	[41, 42]
Pericytic FRCs	Surrounds HEVs PDPN signals to CLEC-2 on platelets to maintain endothelial integrity	PDPN+	Prevents bleeding from HEVs into lymph nodes	[34]
Medullary FRCs	Associated with plasma cells and macrophages	PDPN+	Poorly studied	[4]

1.2.1 Structural Roles

FRCs play crucial roles in secreting extracellular matrix components and forming a cellular meshwork to give the lymph node strength, flexibility and structure [8, 14, 22, 28–30].

While not a focus of this review, as a general characteristic, FRCs secrete a broad array of extracellular matrix components, including collagens and laminins, decorin, biglycan, fibromodulin and vitronectin, to maintain the lymph node structure [8, 14, 22] (Fig. 1.1d).

T zone resident FRCs facilitate leukocyte migration and priming by supporting and secreting a 3D conduit network to maintain the lymph node microenvironment [8, 14, 30–32]. Conduits are microtubules created by FRCs, which secrete constituent

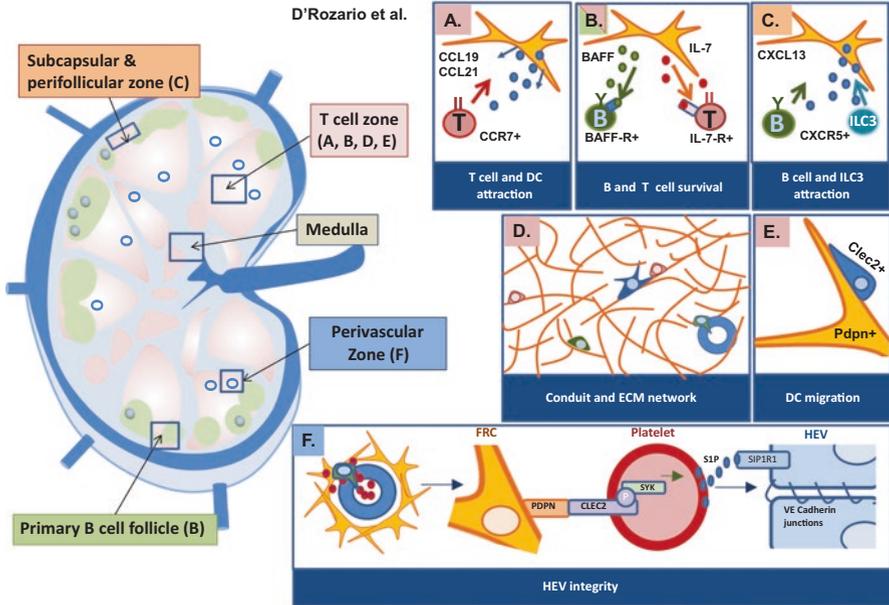


Fig. 1.1 FRC subsets reside in different lymph node niches and fulfil distinct functions. (a) T zone FRCs produce CCR7 ligands CCL19 and CCL21, which promote migration of naïve T cells and dendritic cells [8, 11]. (b) Within primary B cell follicles, B zone FRCs produce BAFF to promote the survival of naïve B cells [24], while within the paracortex, T zone FRCs produce IL-7 to promote the survival of naïve T cells [14]. (c) Within the subcapsular zone, marginal reticular cells produce CXCL13 to interact with innate lymphoid-like cells [39]. In the perifollicular zone close to primary B cell follicles, during inflammation some FRCs inducibly express CXCL13 to facilitate B cell follicle expansion [37]. (d) FRCs within the T zone create the conduit network through secretion of basement membrane and other extracellular matrix components [8, 14, 22, 33]. (e) T zone FRCs drive DC migration via signalling through DC-expressed CLEC2, which binds podoplanin expressed by FRCs [56]. (f) In the perivascular zone, FRCs maintain the blood-lymph barrier by responding to infiltrating platelets. Platelets express CLEC-2, which binds podoplanin on FRCs, delivering a SYK-mediated signalling cascade that results in release of sphingosine-1-phosphate-1 from platelet surfaces, which binds S1P1-receptor on high endothelial venules, stimulating upregulation of VE-cadherin, which tightens endothelial cell junctions and prevents further nontargeted cell and liquid influx from the bloodstream [34]

basement membrane components and ensheath them. They permit low-molecular-weight molecules arriving via lymphatics to permeate quickly into the T cell zone to access resident dendritic cells (DCs) [30, 32, 33]. This allows DCs to rapidly process and present antigen to scanning T cells, permitting speedy initiation of an adaptive immune response. FRCs also surround high endothelial venules (HEVs) where they maintain the blood-lymph barrier by signalling to CLEC2 expressed by infiltrating blood-borne platelets, via the FRC-expressed glycoprotein ligand podoplanin [34]. CLEC-2 signalling induces the release of sphingosine-1-phosphate from the platelet surface, which regulates the binding strength of endothelial cells through VE-cadherin junctions [34].

In response to infection or inflammation, FRCs dynamically regulate lymph node expansion and contraction through expression of podoplanin, which maintains actomyosin contractility under homeostatic conditions and permits relaxation when it binds its ligand CLEC-2, expressed by DCs during inflammation [28, 29]. FRCs also proliferate during inflammation [28, 29, 35]. These dual mechanisms allow the lymph node to dynamically respond to and accommodate changing numbers of lymphocytes during activation and contraction phases of the immune response [28, 29, 35].

These important structural roles for FRCs are briefly discussed here but have been reviewed in detail elsewhere [22, 27].

1.2.2 Interactions with T Cells

1.2.2.1 Provision of Migration and Survival Factors

FRCs exist throughout the paracortical T cell zone; accordingly, interactions with T cells have been most closely studied. Chemotactic factors secreted from paracortical FRCs create the T cell zone by attracting naïve T cells and antigen-presenting cells (APCs) allowing them to initiate an immune response [10, 11, 14, 43]. This occurs through secretion of CCL19 and CCL21, which signal to CCR7 expressed by naïve T cells, leading to their migration through the lymph node [8, 10, 43, 44] (Fig. 1.1a).

T zone FRCs have also been shown to promote the survival and turnover of naïve lymphocytes via the secretion of T cell survival and growth factor IL-7 [14] (Fig. 1.1b). The secretion of this factor regulates and maintains the naïve CD4+ and CD8+ T lymphocyte pool within the lymph node ready for cell priming [14].

These effects of FRCs are particularly relevant to naïve T cells, since activated T lymphocytes within the lymph node are retained and can continue to function when FRCs are depleted [25].

1.2.2.2 Suppressive Tolerance

FRCs are capable of robustly suppressing CD8+ T cell proliferation early after their activation [19–21] (Fig. 1.2a).

Early after activation, T cells secrete inflammatory cytokines interferon gamma (IFN- γ) and tumour necrosis factor alpha (TNF- α), which stimulate FRCs to secrete nitric oxide (NO). NO acts in a paracrine manner on T lymphocytes curbing their proliferation [19–21]. NO is a highly pleiotropic molecule which facilitates many metabolic and immunologic pathways within the body [45]. Activated T cell-derived factors increase NO-producing enzyme nitric oxide synthase 2 (NOS2) mRNA and protein levels in FRCs leading to the release of NO [19–21]. Accordingly, NOS2 $-/-$ FRCs are unable to mediate T cell suppression [19–21]. Cyclooxygenases 1 and 2

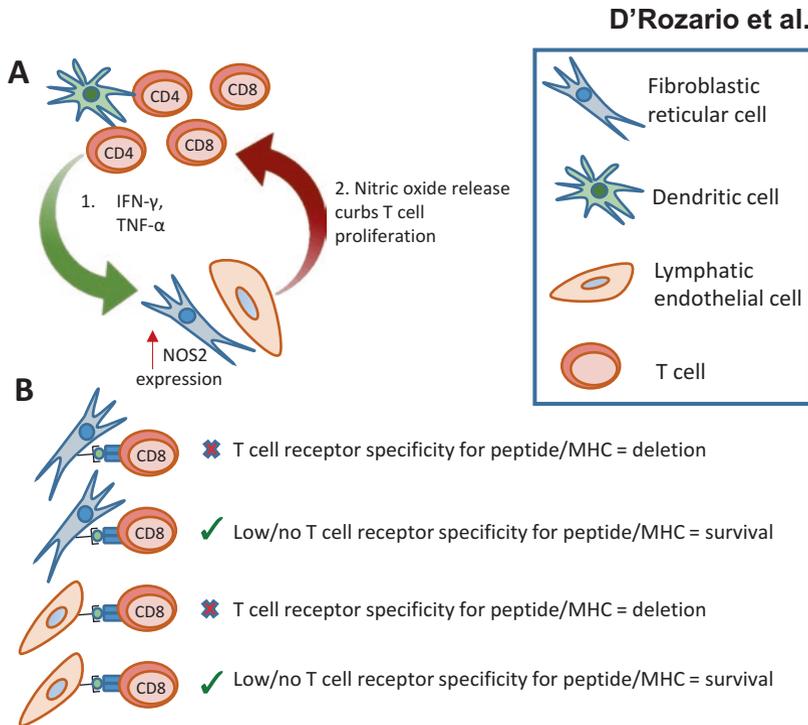


Fig. 1.2 Lymph node stromal cells impose suppressive and deletional tolerance. (a) Newly activated CD8+ T cells produce IFN- γ , TNF- α and an unidentified signal, which induce FRCs to increase expression of enzyme nitric oxide synthase 2 (NOS2) and produce nitric oxide (NO). FRC-derived NO acts on the T cell population to curb proliferation [19–21]. LECs are similarly capable of releasing NO to curb T cell proliferation [20]. (b) LECs and FRCs present endogenously expressed tissue-restricted antigens on MHC class I molecules to CD8+ T cells and delete T cells that respond with sufficient affinity [17, 18]. Similar mechanisms involving MHC class II-dependent antigen presentation are likely to operate for CD4 T cells [23, 26]

(COX1 and 2) in conjunction with NO2 expression have been hypothesised to play a potential role in T cell suppression, though further study is required [21].

This mechanism functions *in vivo* [20], though the immunological reach is still poorly understood.

Lymph node stromal cells may also induce tolerance in CD4+ T cells types through expression of MHC class II and associated antigen presentation pathway molecules under steady state and inflammatory conditions [22, 23, 26]. This theory has been reinforced by the ability of lymph node stromal cells to tolerise CD4+ T cells through the presentation of self-antigens via peptide-MHC class II expression [23, 26] and to induce homeostatic Treg proliferation [23]. *In vitro* data suggest that FRCs, LECs and BECs may acquire MHC II molecules from dendritic cells through cell-to-cell contact [26].

1.2.2.3 Deletional Tolerance

T cell tolerance induction by lymphoid stromal cells was first noted by Lee et al. [13], who showed that CD8+ T cells expressing a TCR reactive to ovalbumin (OVA) were specifically tolerised following interactions with lymph node stromal cells expressing OVA and that this prevented mice that expressed OVA in the gut (iFABP-tOVA) from developing autoimmunity. It was then shown that this response was due to FRCs and that FRCs could directly present self-antigen to T cells via MHC class I [18] (Fig. 1.2b), demonstrating that FRCs were capable of deleting autoreactive T cells and preventing autoimmunity. PD-1^{-/-} T cells or PD-L1-blocking antibodies have been used to interfere with tolerance mechanisms in the iFABP-tOVA model, causing autoimmune enteritis [46].

FRCs were shown to express an array of organ-specific and tissue-restricted antigens [17, 18]. Tissue-restricted antigens (TRAs) are self-antigens native to peripheral tissues and organs and expressed at low levels within lymphoid organs for the purpose of educating the immune system for tolerance induction [47]. A major regulator of TRA expression in the thymus is the autoimmune regulator gene (Aire) [47]. However, in non-haematopoietic lymph node stromal subsets, Aire is not expressed [18]. It has been shown in human and murine tissues that increased expression of the Aire-like *protein DEAF-1* correlates with peripheral tissue antigen (PTA) expression [48]. While other factors may simultaneously exist, these results suggest a role of the *DEAF-1* gene in regulating lymphatic PTA expression, which requires further elucidation.

1.2.2.4 Systemic Effects of Interactions with T Cells

The depletion of FRCs has been shown to significantly attenuate cell-mediated immunity, as FRCs are required for the initiation of antiviral immune responses [25, 49]. In conditional FRC knockout models (DM2 BAC transgenic/FAP-DTR mice), naïve T and B lymphocytes were significantly depleted resulting in poor T and B cell-mediated immune responses during influenza A virus infection [25].

Similarly, the CCL19-Cre × *Ltbr*^{-/-} mouse, which has an abnormal FRC network low in podoplanin, CCL19, CCL21 and IL-7, was unable to clear systemic lymphocytic choriomeningitis virus (LCMV) or mouse hepatitis virus showing a requirement for full FRC maturity [49]. These mice showed a 60–70% depletion of T cells and were unable to clear the viral infections by day 10 compared to control mice [49].

CCL19-Cre × iDTR mice, which are susceptible to inducible depletion of FRCs upon administration of diphtheria toxin, exhibited the loss of naïve CD4+ and CD8+ T cells within the lymph node during FRC ablation, as immunisation of mice with inactive influenza A virus led to an impairment of T cell priming and proliferation, with deterioration of antiviral T cell responses [24].

Furthermore, transplantation of IL-7Cre × R26-EYFP mice lymph nodes into C57BL/6 mice have shown that FRCs play a crucial role in providing IL-7 to initialise successful reformation of lymph node structure after avascular transplanta-

tion [50]. IL-7 derived from FRCs was also shown to promote T cell immunocompetence leading to structural adaptation of the lymph node microenvironment following systemic viral infection [50]. Damage to FRCs in clinical settings, in particular HIV infection, causes profound T cell immunodeficiency independent of viral load [51, 52].

1.2.3 Interactions with B Cells

FRCs in primary B cell follicles produce B cell-activating factor (BAFF) [24], a cytokine which drives the proliferation and maturation of B cells [53]. The production of this cytokine within primary follicles provides a favourable niche for B lymphocytes to develop [24] (Fig. 1.1b). Accordingly, FRC depletion has been shown to reduce the pool of naive B cells within lymph nodes [24, 25].

FRCs in the perifollicular zone have been shown to produce CXCL13 during infection, enabling the B cell follicle to expand and provide a favourable microenvironment for B cell activation and maturation [37, 54] (Fig. 1.1c). Inflammation was initiated by the injection of complete Freund's adjuvant into the ears of mice, and interactions with B cell zone reticular cells were analysed in ear-draining lymph nodes [37]. During systemic inflammation, B cells entered T zone areas of the lymph node in response to CXCL13 expressing B cell zone reticular cells, to expand the B follicle region [37].

1.2.3.1 Systemic Effects of Interactions with B Cells

Creiasco et al. [24] portray the loss of FRCs as detrimental to humoral immunity with immunisation with an inactivated influenza A virus leading to a reduction in influenza-specific immunoglobulin M in conditional FRC knockouts (CCL19-Cre \times iDTR^{fl/fl} mice) compared to control mice. In addition, these mice also exhibited impaired B cell viability and poor B cell follicle organisation, suggesting a systemic FRC importance in humoral immune responses.

In mouse graft-versus-host disease (GVHD) models, CD157+ FRC damage has been shown to impair IgG and IgA humoral immune responses to subcutaneous and oral antigens as B cell follicles are disrupted following FRC reduction [55].

1.2.4 Interactions with Dendritic Cells

Lymph node stroma has been shown *in vivo* to promote DC motility into and within the lymph node via the interactions between FRCs or LECs bearing podoplanin and activated DCs expressing Clec-2 [56]. Using Clec1b (CLEC-2)^{-/-} foetal liver chimeras compared to wild type, it was shown that CLEC-2+ DCs navigate from parenchymal tissues to lymphoid organs by migrating along stromal scaffolds that

display the glycoprotein podoplanin [56]. Activation of CLEC-2 by podoplanin downregulates RhoA activity and phosphorylation of myosin light chains, causing cell spreading, and induces formation of protrusions through Vav signalling and activation of Rac1 [56]. Together these mechanisms promote DC motility across LEC and FRC stromal surfaces to allow antigen-bearing DCs to reach the lymph node and migrate within it in search of antigen-specific T cells.

Recent work highlights an important role for DCs in maintaining FRC survival and proliferation. DCs directly maintain FRC survival through provision of lymphotoxin ligands, which bind lymphotoxin beta receptor (LTbR) on FRCs, which upregulates podoplanin, in turn providing survival stimulus through maintenance of integrin-mediated adhesions [57]. Chyou et al. [58] showed that the initiation of FRC proliferation early in infection does not require DCs but that DCs induce FRCs to upregulate VEGF, which drives expansion of BECs and LECs. Yang et al. [35] revealed that DCs play an important indirect role in initiating FRC proliferation, by inducing naïve lymphocyte trapping within the lymph node early after infection is sensed. Moreover, various chains of MHC class II molecules were shown to become upregulated under inflammation on LECs, FRCs and BECs [22, 23, 26]. This suggests that subsets of LNSCs may be transcribing MHC class II molecules and/or receiving peptides from antigen-presenting cells, demonstrating a further encompassing role of LNSCs in innate immune responses.

1.2.5 Direct Detection of Inflammatory Stimuli and Interactions with Other Immune Cells

FRCs may be involved in the detection of lymph-borne infection or inflammatory signals via the expression of genes associated with pattern recognition toll-like receptors (TLRs) 3 and 4 [18, 22]. As TLRs respond to foreign pathogens by alerting the immune system, this data suggests that FRCs may directly detect viruses and bacteria. This idea has been reinforced by various studies which have documented the (direct or indirect) activation of LECs and FRCs via the usage of viral and bacterial immunostimulants or analogues which interact with TLR 3 and TLR 4 [18, 22, 28, 29, 35, 59].

The upregulation of chemoattractants and regulatory factors associated with the innate immune response has also been identified by transcriptional analysis of LNSCs [22]. FRCs express CXCL1, CXCL10, CCL2, CCL7, IL-33, IL-34, CSF1, CCL5 and CXCL9 and also express receptors for type I and II interferons [22].

1.3 Marginal Reticular Cells

Marginal reticular cells (MRCs) populate the outer regions of the cortex of lymph nodes [39]. They are located deep to the floor of the subcapsular sinus (SCS) and are phenotypically distinct from T zone fibroblastic reticular cells (FRCs) and follicular

dendritic cells (FDCs). MRCs strongly express MAdCAM-1, CXCL13 and RANKL [39, 40]. The latter is an essential cytokine for lymph node development [60, 61]. However, the markers CCL21 expressed by T zone FRCs and CR1/CD35 expressed by FDCs are, respectively, absent or only trace expressed [39], indicating that MRCs are indeed a distinct stromal subset to these populations. Phenotypically similar groups of reticular cells have been found in other secondary lymphoid organs (SLOs), including the spleen and mucosa-associated lymphoid tissues [39, 62]. Contrastingly no similar groups of cells have been found in the tertiary lymphoid organs (TLOs) [5] associated with chronic inflammation.

During organogenesis, lymph nodes develop from accumulations of mesenchyme and haematopoietic cells associated with epithelium or vasculature, known as anlagen [63]. The haematopoietic cells are known as lymphoid tissue inducer (LTi) cells which bear the phenotype CD45+ CD4+ CD3-. LTi cells interact with the mesenchymal cells known as lymphoid tissue organiser (LTo) cells. LTo cells express adhesion molecule (ICAM-1, VCAM-1, MAdCAM-1) and chemokine (CXCL13, CCL19, CCL21) profiles upon stimulation by LTi through their secretion of lymphotoxin (LT)- α 1 β 2 [63, 64]. Subsequently CXCL13 attracts LTi through binding cells at its CXCR5 receptor, propagating a positive feedback loop of development [63, 65–67]. MRCs are thought to be a direct descendent of LTo cells [40]. While yet to be proven, supportive evidence includes their similar molecular phenotypes and the high concentration of LTo cells and RANKL expression in the outer areas of embryonic LNs, which in adult lymph nodes becomes a niche for MRCs [39, 68].

In addition to their embryonic developmental role, the ability of MRCs to give rise to FDCs in the adult lymph node has also been demonstrated. The MRCs exhibit maturation into a transitional phenotype before evolving into phenotypically mature FDCs through a two-step process [41]. FDCs in the spleen arise through other mechanisms, developing from perivascular precursors [69].

Immune-stromal interactions of MRCs are still poorly understood. MRCs are located in various SLOs adjacent to the primary route of antigenic entry, suggesting that they may play a role in the regulation of antigen transportation pathways [39, 68]. In adult mice, MRCs produce CXCL13 to attract CXCR5+ innate lymphoid-like cells type 3 (ILC3), which drive lymph node repair and regeneration after damage [50, 70]. CXCL13 is also a B cell chemoattractant, and it has been hypothesised that MRCs may be involved in the transport of antigens from the SCS into the B follicle or to facilitate the motility of B cells in the outer follicle through their expression of adhesion molecules [39].

The cytokines IL-7 and RANKL are both expressed to high levels by MRCs [39, 68] and are thought to be crucial for lymphoid homeostasis. IL-7 is a naïve T cell survival factor suggesting that it is also involved in regulation of T cell survival. In an *in vitro* murine model, the inoculation of mice with LT β R-Fc resulted in disorganisation of the follicular assembly of the white splenic pulp, and the disappearance of MRC layers, demonstrated by a loss of CXCL13 and RANKL staining [68]. In lymph nodes, LT β R-Fc downregulated CXCL13 but did not alter RANKL staining. This indicates that either RANKL expression in lymph nodes is independent of LT β R-NIK signalling or that another RANKL-expressing cell type is able to compensate for loss of LT β R ligands within the lymph node [68].

Their anatomical placement proximal to the inflow tract of antigens in SLOs, along with cytokine and chemokine expression, suggests that MRCs have an important role in regulating lymphoid function and SLO homeostasis.

1.4 Lymphatic Endothelial Cells (LECs)

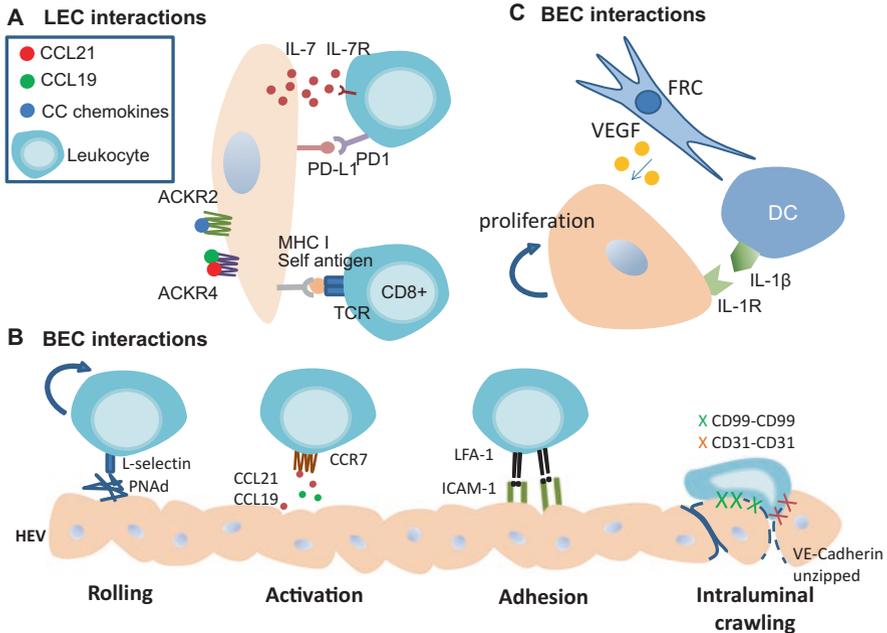
Selectable markers: Gp38+, CD31+, Lyve-1

1.4.1 *Structural and Chemoattractive Role*

LECs create afferent and efferent lymphatic vessels, primarily to allow for the entry of antigen-presenting dendritic cells and soluble antigens into the paracortex of the lymph node [71, 72] and the egress of lymphocytes from the medulla [73]. LECs are also contained within medullary sinuses and line the ceiling (cLECs) and the floor (fLECs) of the subcapsular sinus [72]. It is thought that because of their prime position close to lymph, LECs might also be an early cell type to encounter and present antigens by environmental sampling [74]. LECs from different areas of the lymph node show differing expression of key surface markers: subcapsular LECs are PD-L1hi, ICAM-1hi, MAdCAM-1+ and LTbRlo; medullary LECs are PD-L1hi, ICAM-1hi, MAdCAM-1neg and LTbR+; and cortical LECs are PD-L1int, ICAM-1int, MAdCAM-1neg and LTbR+ [75].

Under inflammatory conditions, LECs direct macrophages and antigen-bearing dendritic cells along lymphatics, between LECs lining the subcapsular sinus, into the lymph node structure. LECs express podoplanin and similar to FRCs are capable of driving DC migration towards and within lymph nodes through signalling to CLEC-2 [56] (see Sect. 1.2.4). They produce CCL21 [22] and are thought to regulate availability of chemokines such as CCL21 and CCL19 in the subcapsular sinus and local parenchymal tissue through expression of scavenging receptors ACKR2 and ACKR4 [76] (Fig. 1.3a). ACKR2 is a scavenging receptor tasked with removing inflammatory cytokines from the cell surface of LECs during inflammation [76]. This allows for suppression of immature DCs and other inflammatory cells and keeps leukocytes from adhering to LECs. ACKR4 is thought to control the distribution of CCL19 and CCL21 to assist DC migration through cognate receptor CCR7, by maintaining optimal availability of these chemokines [76].

While mouse LECs are able to adhere to plastic like FRCs, human LECs are unable to do so indicating a difference in adhesion factors or requirements that is not yet understood [77]. This might be a case of gene downregulation, as many as 50% of LEC-defining genes were found to be silenced under culture conditions compared to freshly isolated cells [78].



1.4.2 Interactions with T Cells and DCs

1.4.2.1 Provision of Survival Factors

LECs are a robust source of IL-7 and together with FRCs, which also produce IL-7, likely to be important regulators of T cell homeostasis [79] (Fig. 1.3a). IL-7 has proliferative and anti-apoptotic signalling abilities and is important for T cell

survival in SLOs. In vitro experiments of co-cultures of LECs with T cells or T cells with conditioned media from LECs show an improved ability to promote T cell survival compared to a similar setup with the addition of anti-IL7 neutralising antibody [50].

1.4.2.2 Suppressive Tolerance

LECs are capable of suppressing the proliferation of newly activated CD4 and CD8+ T cells through the production of nitric oxide, similar to FRCs [20] (Fig. 1.2a). Under inflammatory conditions, but in the absence of infection, LECs suppress maturation of DCs, reducing expression of CD86 and their ability to prime CD8+ T cells [80]. This occurred through binding between ICAM-1 on LECs and Mac-1 on DCs and is hypothesised to reduce the risk of immune priming under inflammatory conditions in the absence of infection [80].

LECs show upregulation of surface MHC class II 18 h after initiation of an inflammatory response [22]. This may contribute to CD4 T cell tolerance through increased antigen presentation. This upregulation is IFN γ dependent [26]. LECs are capable of transiently acquiring peptide-MHC class II from DCs in vivo and in vitro, directly proportional to the number of DCs present [26]. In the same study, LECs were shown to promote apoptosis of CD4+ T cells in an antigen-specific fashion [26].

Recently, Hirosue et al. [74] demonstrated that LECs can absorb exogenous OVA and will process and cross-present the OVA-derived SIINFEKL peptide fragment to CD8+ T cells in vitro [74] (Fig. 1.3a). LECs also upregulated PD-L1, which signals to PD-1 expressed by T cells and is a well-known cause of T cell exhaustion under conditions of prolonged inflammation (Fig. 1.3a). OVA-specific CD8+ T cell activation was impaired; T cells stimulated by LECs made less IL-2 and upregulated CTLA-4 earlier than those activated by DCs [74].

1.4.2.3 Deletional Tolerance

Similar to FRCs, LECs within lymph nodes express a variety of peripheral tissue-restricted antigens (PTAs), including Deaf-1 controlled Ins2 and Ppy [48, 81], though FRCs and LECs do not express identical arrays of PTAs [17, 18]. PTA expression by tolerance-inducing cells, such as LECs, is pivotal to delete T cells reactive to endogenous antigens expressed in relatively few tissues. Cohen et al. [17] showed using an endogenous melanocyte-specific self-antigen derived from tyrosinase that LECs directly presented self-antigen to Tyr-specific CD8+ T cells, deleting those that respond and purging the repertoire of autoimmune clones (Figs. 1.2b and 1.3a). In contrast, LECs indirectly induce CD4+ T cell anergy by presentation of PTAs to DCs [82], showing that LECs utilise different mechanisms of tolerance induction for CD4+ and CD8+ T cells.

In the thymus, Aire controls the expression of PTAs, but in lymph node stromal cells, Aire is not expressed [18]. However, studies in NOD mice demonstrate that PTA genes that are Aire-controlled in the thymus, such as *Ambp*, *Fgb* and *Ppy*, are regulated by the transcription factor Deaf-1 in the lymph node [48], which is expressed by LECs as well as FRCs [18]. During the progression of diabetes, alternative splicing of Deaf-1 occurs reducing PTA expression in mice and humans, but the effect of alternative splicing and PTA expression with respect to development of diabetes and other diseases is yet to be determined [48].

LECs do not express CD80, CD86, OX40L, 4-1BBL or CD70. These are essential molecules to drive accumulation of activated T cells, and their lack of costimulatory molecule expression may help account for their ability to delete naïve autoantigen expressing T cells. However, LECs express high levels of PD-L1, a molecule associated with deletion of tolerance-specific CD8+ T cells [17, 75].

Lymph node LECs were unique in their high expression of PTAs and PD-L1, compared with LECs in peripheral tissues such as the diaphragm and colon, showing that the lymph node microenvironment is uniquely specialised for tolerance induction [75].

1.5 Blood Endothelial Cells (BECs)

Selectable markers: Gp38-, CD31+, ICAM-1+

1.5.1 *Transendothelial Migration*

BECs facilitate the migration of naïve lymphocytes into lymph nodes by forming specialised postcapillary venules known as high endothelial venules (HEVs) [3]. BECs comprising HEVs show a distinctive cuboidal morphology supported by a basement membrane and have been shown to play a specialised role in allowing lymphocyte entry to SLOs through the process known as diapedesis, transendothelial migration or leukocyte extravasation (Fig. 1.3b). Migration into lymph nodes does not require inflammation, but bears similarities with migration of leukocytes to inflamed sites [83]. HEVs act as gatekeepers for lymph nodes by creating pockets holding newly arrived lymphocytes until space in the parenchyma becomes available, granting entry at a rate proportionate to egress from the lymph node [9].

During cell circulation, naïve T and B cells enter the medulla by squeezing between tightly adherent endothelial cells forming high endothelial venules (HEVs). The process of slowing down and breaching the endothelial barrier involves well-characterised interactions including leukocyte rolling, activation, adhesion and intraluminal crawling [84], which involve targeted interactions between endothelium and T cells [85] (Fig. 1.3b).

L-selectin is a primary mediator of rolling and loose attachment to HEVs. During inflammation, cytokines also upregulate expression of P-selectin and E-selectin by HEVs [3]. L-selectin binds peripheral node addressin (PNAd), referring to a group of sialomucins including CD34, podocalyxin, endomucin and nepmucin (mice and humans), as well as Glycam-1 (mice only). PNAd is expressed only by BECs comprising HEVs [58]. These glycoproteins are heavily sialylated, fucosylated and sulphated, in part through the activity of HEV-restricted GlcNAc-6-sulfotransferase [86]. Next, lymphocytes undergo integrin-mediated arrest primarily involving LFA-1 [87], binding endothelial ICAM-1 molecules, which cluster beneath the T cell to anchor it. Signalling to ICAM-1 also initiates intracellular processes that prepare endothelial cells for transendothelial migration. Next, PECAM (CD31) and CD99, each expressed by both leukocytes and endothelial cells with homophilic affinity, arrest leukocytes near the junction of adjacent endothelial cells [84]. Loss of CD31 arrests leukocytes at the junction, while loss of CD99 arrests leukocytes after they have begun to enter the junction [84]. Lastly, adherens junctions joining endothelial cells are disassembled through phosphorylation of VE-cadherin, which occurs downstream of ICAM-1 signalling and SHP2 recruitment [84] (Fig. 1.3b).

In mice, but not humans, BECs produce CCL21, which assists with arrest of rolling lymphocytes by binding G protein-coupled receptor CCR7 expressed by naïve T cells [3, 88] and may drive lymphocyte migration across the HEV barrier into the paracortex [89]. During inflammation, lymphatics bring an influx of pro-inflammatory chemokines including CCL2 and CXCL9, which are transported through conduits to the HEV lumen to increase the influx of T cells, B cells, NK cells and monocytes [3].

HEV barriers into the lymph node are thought to be regulated by atypical cytokine receptor 1 (ACKR1) that is responsible for the transport of chemokines in vivo. However, more experimental evidence is needed to substantiate this theory, as knockout ACKR1 knockout mice exhibit a varied phenotype [76].

1.5.2 BEC Proliferation and Homeostasis

Mice with endothelial cell-specific ablation of LT β R (VE-cadherin-Cre \times Ltbr^{fl/fl}) showed reduced lymph node formation, altered HEV phenotype and a reduction of lymphocytes entering lymphatic organs [90]. Endothelial cells lost their cuboidal shape and polarisation with reduced ICAM-1 expression. An overall reduction in homing of lymphocytes was recorded, but T cell motility once inside the lymph node was not affected [90]. BECs have also been shown to increase in number during inflammation and are responsible in maintaining vascular integrity and haemostasis [34, 35, 91, 92].

Like FRCs and LECs, BECs are also important for network remodelling of the lymph node. Stromal proliferation is common for FRCs, LECs and BECs upon infection, and FRCs and HEV BECs appear to begin proliferating simultaneously as early as 2 days postinfection [58]. FRCs are the highest producers of vascular endo-

thelial growth factor (VEGF) and are likely to contribute to growth of BECs through this molecule [58] (Fig. 1.3c).

DCs also regulate HEV phenotype and function (Fig. 1.3c). Webster et al. [93] showed that mice depleted of DCs (CD11c-DTR mice) showed a significant decrease in lymph node size and endothelial cell proliferation after injection of OVA/CFA, compared to controls. In addition, RAG1^{-/-} mice that exhibit decreased cellularity and basal levels of endothelial cells still showed size increase of lymph nodes upon injection of bone marrow-derived DCs [93]. IL-1 β is thought to play a partial role in inducing endothelial cell proliferation by DCs, but not T and B cells, though other factors are yet to be identified [94]. Subsequent studies also show that T and B cells, while dispensable in initial endothelial proliferation, play a major role in subsequent maintenance and expansion of the lymph node [58].

Recent transcriptomic analysis has revealed the need for caution when using cultured BECs and LECs. The transcriptional profile of cultured BECs resembles that of LECs and might lead to misidentification during experimental procedures [78]. Specifically, more than 65% of genes selectively expressed by BECs *in vivo* are downregulated during culture, including MHC class II, E-selectin and ICAM-1 [78]. However, LECs maintain podoplanin and CD146 gene expression in culture, allowing differentiation from BECs [78].

1.6 Follicular Dendritic Cells (FDCs)

Selectable markers: CD35+, CD21+ FDC M1+, CXCL13+ ICAM1+ VCAM1+ BAFF+

1.6.1 Interactions with B Cells

FDCs are non-haematopoietic stromal cells contained in B cell follicles within lymphoid organs, where they are able to capture incoming antigen and store it long-term for presentation to B cells while also producing the B cell survival factors BAFF and IL-6 [42, 95–97]. FDCs play critical roles in the formation of efficient germinal centres and efficient somatic hypermutation of B cells and subsequent production of high-affinity antibodies [98, 99]. B cells can also directly capture antigen from FDCs [100], and elimination of FDCs eliminates germinal centres [98, 99, 101, 102].

FDCs are the major source of CXCL13 in lymph nodes, a chemokine which plays a crucial role in organising B follicles and the formation of germinal centres [43, 103, 104]. CXCL13 also signals to B cells and T helper cells leading them towards the follicles [43], and it enhances B cell activation [54]. FDCs retain intact antigen for extended periods (up to 12 months), which facilitates germinal centre maintenance and B cell somatic hypermutation [42].

Activated B cells migrate to the border of the follicle to present antigen to T helper cells, which provide essential costimulatory signals. B cells receiving help then migrate to the follicle's centre to proliferate and undergo hypermutation and are then subjected to selection by FDCs on the basis of recognition of antigens displayed by FDCs. Activated B cells interact with antigen presented on FDCs in a process called affinity selection, after which they progress to one of several fates (to proliferate further, class-switch or become plasma or memory B cells), while non-responding B cells become apoptotic [42].

FDCs are of enormous immunological relevance; accordingly there is an extensive literature on FDCs (see Heesters et al. [42]) that unfortunately cannot be discussed at length in this review.

1.7 Double-Negative Stromal Cells and Podoplanin-Negative Pericytes

Selectable markers: Gp38-, CD31-, ITGA7+, calponin-1

1.7.1 Identification and Characterisation

GP38-CD31 double-negative (DN) stroma represent approximately 10–20% of non-haematopoietic lymph node stromal cells [14, 18, 59]. Until recently the lineage, location and function of these cells were undescribed.

Malhotra et al. [22] used transcriptomics of sorted lymph node stromal subsets to identify a high similarity between the DN cells and fibroblastic reticular cells (FRCs), including similarities in chemokine, cytokine and growth factor expression. FRCs showed higher expression of CCL19 and CCL21 [22], though this may have been due to the heterogenous nature of the DN pool, as not all DN cells were likely to be fibroblastic in origin. A noteworthy difference was that IL-7 expression was restricted to FRCs and lacking in DN cells, while DN cells showed higher expression of the genes responsible for structure and contractile functions including higher expression of actin subtypes and myosin chain genes, which usually control smooth muscle contraction [22].

Accordingly, approx. 50% of DN cells were identified as a specialised subset of myofibroblastic pericytes, localised using specific expression of calponin-1 and integrin $\alpha 7$ [22]. Both calponin-1 and integrin $\alpha 7$ contribute to muscular function; calponin-1 is a specific actin protein that regulates force in contractile cells, especially smooth muscle cells [105], and integrin $\alpha 7$ connects the extracellular matrix with muscle fibres [106]. Staining with these antibodies identified this subset of double-negative cells around certain vessels in the cortex and the medulla [22]. The subset was named integrin alpha-7+ pericytes [22]. Their function is undescribed,

but shared expression of many immunologically relevant molecules with FRCs may suggest similar functions. The other 50% of cells comprising the DN subset are undescribed.

1.8 Conclusions

Lymph node stromal cells form crucial roles in maintaining lymph node structure and homeostasis and have evolved to become major contributors to the immune system via their cellular interactions. This area of study has instigated a paradigm shift in the study of tolerance and has increased our understanding on immune cellular interactions. Evidence of biologically significant crosstalk occurring between stromal cells and the immune system continues to emerge, including interactions that rebuild the immune system after damage or prevent autoimmunity. These findings continue to demonstrate the importance of further research into stroma from lymphoid organs.

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