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ABSTRACT

Afferent lymphatic vessels transport leukocytes and soluble molecules from the periphery to lymph nodes. These vessels are lined with lymphatic endothelial cells (LEC), which are also present in lymph node sinuses. Lymph nodes are centers for the initiation of immune reactions, where antigen presenting cells present foreign antigens to naïve lymphocytes. Lymph-borne antigens are sorted based on their size in the subcapsular sinus (SCS) of the lymph node and only small antigens get direct access to the lymph node parenchyma. Lymphocytes migrate constantly into lymph nodes in search of foreign antigens. After searching for their cognate antigens, lymphocytes leave the nodes by egressing at the lymph node medullary sinus and the efferent lymphatics.

LECs are known to regulate cell entry into lymph nodes as well as lymphocyte egress at the medullary sinus. The differences between the two populations of lymph node LECs, namely the afferent and efferent lymphatics, have not been thoroughly investigated. My doctoral studies describe two new molecules expressed in the afferent and efferent lymphatics (Msr1 and Robo4) and their role in lymphocyte migration. We also investigated how large antigens and antibodies get access into the lymph node parenchyma. Msr1 and Robo4 regulate lymphocyte migration and turnover in peripheral lymph nodes and Peyer's patches. Moreover, we discovered that LECs transcytose large antigens and antibodies instantly into the lymph node parenchyma. These studies broaden the current view of LECs as regulators of cell and molecular trafficking at the lymph node sinuses. Our results also suggest that the effective delivery of lymph-borne antibodies into lymph nodes may be therapeutically harnessed.

KEYWORDS: lymphatic endothelial cells, lymph node, lymphocyte migration, Msr1, Robo4, antibody transcytosis

TURUN YLIOPISTO
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TIIVISTELMÄ

Imusuonet kuljettavat valkosoluja sekä liukoisia molekyylejä perifeerisistä kudoksista imusolmukkeisiin. Lymfaattiset endoteelisolut verhoavat sekä suonien että imusolmukkeen onteloiden sisäpintoja. Imusolmukkeet ovat tärkeitä keskuksia, joissa elimistön immuunireaktiot saavat alkunsa. Antigeenejä esittelevät valkosolut esittelevät vieraat molekyylit lymfosyyteille, mikä saa aikaan niiden aktivoitumisen. Imunesteessä olevat antigeenit lajitellaan koon mukaan imusolmukkeen subkapsulaarisinuksessa ja vain pienillä molekyyleillä on pääsy imusolmukkeen sisäosiin. Lymfosyytit kiertävät jatkuvasti imusolmukkeissa etsien vieraita antigeenejä ja poistuvat imusolmukkeesta medullaarisinuksen vieviä imusuonia pitkin.

Lymfaattiset endoteelisolut säätelevät valkosolujen pääsyä imusolmukkeeseen sekä niiden pois lähtöä medullaarisinuksessa. Imusolmukkeessa olevien tuovien ja vievien endoteelisolujen eroja ei ole aikaisemmin kuvattu. Väitöskirjassani tutkin kahden uuden lymfaattisissa endoteelisoluissa ilmennettävän molekyylin (Msr1, Robo4) merkitystä valkosoluliikenteen säätelyssä. Selvitimme myös, miten suuret antigeenit sekä vasta-aineet pääsevät imusolmukkeen sisäosiin. Sekä Msr1 että Robo4 säätelevät lymfosyyttien kulkua niin perifeerisissä kuin suoliston pinnan imusolmukkeissa. Lymfaattiset endoteelisolut myös kuljettavat aktiivisesti suuria antigeenejä sekä vasta-aineita imusolmukkeen sisäosiin. Väitöskirjani tulokset laajentavat käsitystämme lymfaattisten endoteelisolujen merkityksestä imusolmukkeen liikenteen säätelijöinä. Tutkimukseni tuloksia vasta-aineiden kulkemisesta imuteitä pitkin imusolmukkeisiin voidaan myös hyödyntää terapeuttisesti.

AVAINSANAT: lymfaattiset endoteelisolut, imusolmuke, lymfosyyttiliikenne, Msr1, Robo4, vasta-aineiden transsytoosi

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Abbreviations

ATP	Adenosine triphosphate
BABB	Benzyl alcohol and benzyl benzoate
BEC	Blood vessel endothelial cell
Cav1	Caveolin 1
cDC	Classical DC
CLEC-2	C-type lectin-like receptor 2
CLEVER-1	Common lymphatic endothelial and vascular endothelial receptor-1
COUP-TFII	Chicken ovalbumin upstream transcription factor II
CSF1	Colony stimulating factor 1
DC	Dendritic cell
DAPI	4’,6-diamidino-2-phenylindole
E	Embryonic day
ECM	Extracellular matrix
EEA1	Early endosome antigen 1
ELISA	Enzyme-linked immunosorbent assay
FAE	Follicle-associated epithelium
FcRn	Neonatal Fc receptor
FCS	Fetal calf serum
FDC	Follicular dendritic cell
FITC	Fluorescein isothiocyanate
FRC	Fibroblastic reticular cell
GALT	Gut-associated lymphoid tissue
GC	Germinal center
GlyCAM-1	Glycosylation-dependent cell adhesion molecule-1
GPCR	G protein coupled receptor
HBSS	Hanks’ buffered salt solution
HEV	High endothelial venule
ICAM-1	Intercellular adhesion molecule 1
IFR	Interfollicular region
IL-7	Interleukin 7
JAM	Junctional adhesion molecule

KO	Knockout
LPS	Lipopolysaccharide
MAdCAM-1	Mucosal vascular addressin cell adhesion molecule 1
MCM	Medullary cord macrophage
MDC	Monodansylcadaverine
migDC	Migratory dendritic cell
MHC	Major histocompatibility complex
MLN	Mesenteric lymph node
MRC	Marginal reticular cell
MSM	Medullary sinus macrophage
Msr1	Macrophage scavenger receptor 1
LEC	Lymphatic endothelial cell
LFA-1	Lymphocyte function-associated antigen 1
LPA	Lysophosphatidic acid
$LT\alpha_1\beta_2$	Lymphotoxin-$\alpha_1\beta_2$
LTβR	Lymphotoxin-β receptor
LTi	Lymphoid tissue inducer cell
LTo	Lymphoid tissue organizer cell
OVA	Ovalbumin
PECAM-1	Platelet endothelial cell adhesion molecule
PFA	Paraformaldehyde
PLVAP	Plasmalemma vesicle associated protein
PNAd	Peripheral node addressin
Prox1	Prospero homeobox protein 1
RANK	Receptor activator of nuclear factor κ B
RANKL	Receptor activator of nuclear factor κ B ligand
Robo4	Magic roundabout
SCS	Subcapsular sinus
SED	Subepithelial dome
SSM	Subcapsular sinus macrophage
Tfh	T follicular helper cell
Treg	T regulatory cell
VAP-1	Vascular adhesion protein 1
VCAM-1	Vascular cell adhesion molecule 1
VE-cadherin	Vascular endothelial cadherin
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VLA-4	Very late antigen-4
WT	Wild type

List of Original Publications

This dissertation is based on the following original publications, which are referred to by their Roman numerals:

I Iftakhar-E-Khuda I., Fair-Mäkelä R., Kukkonen-Macchi A., Elima K., Karikoski M., Rantakari P., Miyasaka M., Salmi M. and Jalkanen S.: Gene-expression profiling of different arms of lymphatic vasculature identifies candidates for manipulation of cell traffic. *Proc Natl Acad Sci U.S.A.,* 2016; 113(38):10643–10648.

II Fair-Mäkelä R., Ugur M., Iftakhar-E-Khuda I., Kähäri L., Kukkonen-Macchi A., Brenyah H.-M., Elima K., Miyasaka M., Pabst O. and Jalkanen S.: Robo4 contributes to the turnover of Peyer's patch B cells. *Mucosal Immunol,* 2020; 13:245–256.

III Kähäri L.*, Fair-Mäkelä R.*, Auvinen K.*, Rantakari P., Jalkanen S., Ivaska J. and Salmi M.: Transcytosis route mediates rapid delivery of intact antibodies to draining lymph nodes. *J Clin Invest,* 2019; 129(8):3086–3102.
* These authors contributed equally

1 Introduction

Immune cell migration is vital for our health. Our immune system is constantly surveilling the body for pathogens, foreign antigens and other malignant cells for it to target and eliminate. In order to find these harmful agents, immune cells migrate throughout the body via the blood and lymphatic vasculature. The lymphatic system contains lymph nodes that are attached to one another by lymphatic vessels. These secondary lymphoid organs are reactive centers, where adaptive immune responses are initiated. Lymphocytes migrate into lymph nodes in order to recognize foreign antigens. When encountering them, lymphocytes become activated and are trained to attack and eliminate these agents in protection of the host. For successful immune reactions to take place, migration of immune cells must be tightly regulated. In addition, the transport of foreign antigens to sites of immune recognition has to be carefully guided. (von Andrian and Mempel, 2003; Randolph et al., 2017)

Naïve lymphocytes migrate into lymph nodes mainly via the blood vasculature, whereas antigens, antigen-presenting cells and other lymphocyte subpopulations migrate into lymph nodes via lymphatic vessels. Not only does the lymph node harbor immune cells, it also acts as a filter that determines which molecules present in lymph are able to enter the node. Small soluble molecules drain into the lymph node parenchyma within a specialized reticular conduit system, while large antigens only get access to the lymph node within migratory dendritic cells (DC). Antigens, DCs and other lymphocytes migrating to the lymph node via the afferent lymphatics drain into the lymph node subcapsular sinus (SCS), which is lined by lymphatic endothelial cells (LEC). LECs regulate the entry of immune cells into and out of the lymph node. After naïve lymphocytes have searched for their cognate antigens, they leave the lymph node through the medullary sinus, also lined by LECs, and enter the efferent lymphatics. (von Andrian and Mempel, 2003; Girard et al., 2012; Miyasaka and Tanaka, 2004)

LEC-regulated cell migration has been studied, yet the differences between the afferent and efferent lymphatics of the lymph node have not been thoroughly described. My doctoral studies set out to study the molecular differences between the LECs of the SCS and lymphatic sinus of the lymph node, representing the

afferent and efferent arm of the lymphatics, respectively. The cells were subjected to a genome-wide microarray to reveal molecular differences. Two molecules were selected for functional studies; namely, macrophage scavenger receptor 1 (Msr1) and magic roundabout (Robo4). Msr1 was shown to be expressed by the SCS LECs, and we discovered it to regulate lymphocyte migration via the lymphatics into the peripheral lymph node. Robo4 was discovered to be expressed by LECs of the medullary sinus as well as in the lymphatics of Peyer's patches. We were able to describe a function for Robo4 in retaining a subset of naïve B cells in the Peyer's patch suggesting a role for Robo4 in B cell turnover.

Our studies also revealed a new mechanism for the filtering function of the lymph node. We discovered that large lymph-borne antigens and antibodies get instant access into the lymph node. Our studies indicate that LECs are the cell type actively transporting antibodies and other large antigens from the SCS into the lymph node parenchyma. This transport could be harnessed for immunomodulation of the immune response. The presented study reveals new mechanisms for LECs in regulating immune cell migration as well as antigen transport, both of which are fundamental for the successful function of our immune system.

2 Review of the Literature

2.1 The lymphatic system

The lymphatic system is a linear, open-ended network of lymphatic vessels connecting lymphoid organs to each other. In addition to lymphatic vessels, the lymphatic system comprises of the following organs: lymph nodes, the thymus, tonsils, spleen and Peyer's patches. Protein-rich filtrate of plasma leaks out of the capillaries into the surrounding interstitium, where lymphatic capillaries absorb it creating lymph. Lymph is transported within the lymphatic vessels through lymph nodes and eventually to the thoracic duct, which connects to the subclavian vein. This one-directional movement of lymph ensures the careful filtering of its content by lymph nodes and the returning of extracellular fluid back to the bloodstream. Key functions of the lymphatic system are the uptake and recirculation of extracellular fluid as well as immune cells to lymphoid organs. Impairment in the recirculation of fluid leads to lymphedema, one of the main pathologies of the lymphatic system. The lymphatic system is also associated with other major pathologies such as atherosclerosis and cancer. (Choi et al., 2012; Moore Jr. and Bertram, 2018; Randolph et al., 2017)

2.1.1 Early development of lymphatic vessels

The lymphatic system develops independently of the blood vasculature. LECs can form by budding from existing veins, or they may differentiate from mesechymal-cell derived precursors. (Ulvmar and Mäkinen, 2016) Around embryonic day (E) 9.5, the prospero homeobox protein 1 encoding gene *Prox1* is expressed by a subpopulation of vascular endothelial cells in the cardinal vein, which commits these cells to the lymphatic lineage and leads to the formation of early lymph sacs. The whole lymphatic system gradually develops from these primitive sacs. Mice lacking *Prox1* completely fail to develop a lymphatic system as LEC budding from the cardinal vein is prematurely arrested. (Wigle and Oliver, 1999; Wigle et al., 2002) Lineage studies with *Prox1* reporter mice have confirmed the venous origin of LECs (Srinivasan et al., 2007). Expression of *Prox1* is regulated by at least two other transcription factors: *Sox18* and the orpan nuclear receptor *Coup-TFII*

(*chicken ovalbumin transcription factor II*). Both of the transcription factors can directly activate *Prox1*, regulate its expression and thus contribute to committing cells to the lymphatic lineage. (François et al., 2008; Srinivasan et al., 2010)

Although *Prox1* has been termed the master regulator for lymphatic development, other molecules are also essential in the process. Vascular Endothelial Growth Receptor 3 (VEGFR-3) is expressed in lymphatic vessels during development (Kaipainen et al., 1995), and signaling through this receptor is required for the development and maintenance of the lymphatic vascular system (Mäkinen et al., 2001). One of its ligands, Vascular Endothelial Growth Factor C (VEGF-C), is expressed as early on as E8.5 in the region, where the first lymph sacs form. VEGF-C is required for the sprouting of *Prox1*-expressing LECs from the cardinal vein allowing them to produce the primitive lymph sacs. (Karkkainen et al., 2004)

Lymphatics may also develop from non-endothelial cells. A subpopulation of dermal lymphatics was shown to have a non-venous origin, as skin lymphatics were visible after depletion of the blood vasculature (Martinez-Corral et al., 2015). Also, progenitor cells of the hemogenic endothelium, i.e. endothelial cells in the vascular endothelium that can differentiate into hematopoietic cells, proved to be the source for part of the mesenteric lymphatic vessels (Stanczuk et al., 2015) as well as a subset of cardiac lymphatics (Klotz et al., 2015). These studies, which concentrated on organ-specific lymphatics, confirm that lymphatics may develop from several different origins.

2.1.2 Lymphatic vessel structure

In the periphery, lymphatic vessels start out as capillaries or initial lymphatics designed for the uptake of fluids. Gradually the small capillaries form larger collecting vessels in a tree-like structure. The larger vessels are responsible for the transport of lymph and cells to the blood circulation. The collecting lymphatics finally merge into the thoracic duct, which drains lymph back into the blood circulation at the subclavian vein. (Randolph et al., 2005)

Structurally, the initial lymphatics are thin, blind-ended vessels that are composed of a single endothelial cell layer. Unlike blood vessel capillaries, initial lymphatics have a discontinuous basement membrane and no surrounding pericytes or smooth muscle cells. Therefore, lymphatic capillaries connect to the extracellular matrix (ECM) with anchoring filaments. (Leak and Burke, 1968; Paupert et al., 2011) Endothelial cells of initial lymphatics are interconnected by special button-like junctions, which allows fluids to flow into the capillaries (Baluk et al., 2007). The initial lymphatics grow into precollector vessels surrounded by a poorly defined basement membrane, and these vessels further progress into the

collecting lymphatics. In comparison to the initial lymphatics, collecting lymphatics are surrounded by a basement membrane, pericytes and smooth muscle cells, and they exhibit continuous zipper-like junctions between endothelial cells. In addition, several lymphatic valves are present in precollecting and collecting lymphatics to aid fluid flow. (Baluk et al., 2007; Ryan, 1989)

2.1.2.1 Lymphatic endothelial cells

LECs line the lumen of lymphatic vessels similar to blood vessel endothelial cells (BEC) lining the lumen of blood vessels. LECs resemble BECs in several ways, and only research in the late 1990s discovered several lymphatic markers allowing studies to concentrate on the lymphatics. The differences between LECs and BECs as well as differences between the lymphatic and blood vasculature have been summarized in Table 1 and will be further discussed here.

One of the early markers discovered for LECs was VEGFR-3 (Kaipainen et al., 1995), which binds VEGF-C and VEGF-D (Achen et al., 1998; Joukov et al., 1996). Around the same time, expression of LYVE1 (lymphatic vessel endothelial hyaluronan receptor 1, Banerji *et al.*, 1999) and podoplanin (Breiteneder-Geleff et al., 1999) was reported to be specific for the lymphatics. Prox1 was discovered to regulate the lymphatic lineage in developmental studies (Wigle and Oliver, 1999). It has been shown to activate other lymphatic genes, such as VEGFR-3, and to downregulate genes associated with the blood vasculature when expressed in human cell lines. Thus, Prox1 has rightfully earned its title as master regulator. (Hong et al., 2002; Petrova et al., 2002) Neuropilin-2, a member of the non-kinase neuropilin receptors, is also expressed by the lymphatics, where it is important for the development of small lymphatic vessels and capillaries (Yuan et al., 2002).

As most LECs are derived from the same origin as BECs, there are several common markers that are shared by the two vasculatures. Platelet endothelial cell adhesion molecule (PECAM-1, CD31) and VE-cadherin, both expressed in tight junctions connecting endothelial cells to each other, are known to be expressed by both vasculatures. (Ulvmar and Mäkinen, 2016) PLVAP (Plasmalemma vesicle associated protein), the antigen for the antibody PAL-E and MECA-32, also stained LECs in the lymph node in addition to its strong staining on blood vessels (Hallmann et al., 1995; Rantakari et al., 2015). Moreover, other molecules such as Common lymphatic endothelial and vascular endothelial receptor-1 (CLEVER-1) and CD73 are expressed in both vasculatures variably in different tissues (Ålgars et al., 2011; Irjala et al., 2003).

Despite being similar in many ways, LECs and BECs differ significantly in their energy metabolism. Although BECs are in direct contact with the oxygen they transport, they generate ATP via glycolysis, which is crucial for the rapid

expansion of endothelial cells *in vitro* (Parra-Bonilla et al., 2010). Defects in the glycolysis pathway also have an impact on BEC migration resulting in impaired blood vessel sprouting (De Bock et al., 2013). In contrast, LEC energy metabolism relies on fatty acid oxidation, which regulates LEC proliferation and differentiation by the direct binding of Prox1 to CPT1A, a rate-controlling enzyme of fatty acid oxidation (Wong et al., 2017).

Table 1. Summary of the features of the lymphatic and blood vasculature and their significant differences as discussed in the text.

	LYMPHATIC VASCULATURE	BLOOD VASCULATURE
MARKERS	VEGFR-3, Prox1, LYVE1, podoplanin, PECAM-1, PLVAP, VE-cadherin	PECAM-1, PLVAP, VE-cadherin
JUNCTIONS	Button-like, zipper	Zipper
METABOLISM	Fatty acid oxidation	Glycolysis
VESSEL SUPPORTING STRUCTURES	Initial lymphatics: anchoring filaments Collecting lymphatics: basement membrane, pericytes, smooth muscle cells	Basement membrane, pericytes, smooth muscle cells
FUNCTION	Maintaining fluid homeostasis, conveying immune cell migration, lipid uptake, nutrient transport	Delivery of oxygen, nutrients, waste; transport of immune cells, barrier function
MIGRATING IMMUNE CELLS	Dendritic cells, effector and memory lymphocytes, macrophages	All leukocytes

2.1.3 Function of the lymphatic system

Lymphatic vessels are present in nearly all tissues throughout the whole body. The lymphatic system has three major roles under homeostatic conditions: (1) maintaining interstitial fluid balance, (2) conveying antigen and immune cell trafficking throughout the body and (3) absorbing lipids and nutrients in the intestine. (Choi et al., 2012) The role of the lymphatics in antigen and immune cell migration will be described in detail under sections 2.2.3 and 2.3.

2.1.3.1 Regulation of fluid homeostasis

A protein-rich filtrate of plasma leaks out of blood vessel capillaries into the surrounding tissue. Lymph is produced when the interstitial pressure exceeds the pressure of the initial lymphatics forcing the protein-rich fluid from the tissue into the lymphatic vessels. Increased interstitial pressure causes tension, which results

in opening of the button-like junctions of the initial lymphatics. This allows fluid to flow into the vessel. After the interstitial fluid enters the initial lymphatic vessels, the pressure dynamics shift, and the greater pressure in the lymphatic vessel leads to the closing of the endothelial cell flaps. This in turn prevents the fluid from flowing back into the surrounding tissue. (Sabine et al., 2016; Zawieja, 2009)

Low pressure in the peripheral lymphatics does not support the 'flowing' of lymph towards the bloodstream. Instead, lymph is actively pumped along the vessels. As the interstitial pressure is close to or even lower than the atmospheric pressure, a pressure gradient favoring the production of lymph must be formed by the tissue or the lymphatic vessel itself. Extrinsic factors, such as muscle contraction and breathing, in addition to intrinsic factors, e.g. the contraction of the lymphatic vessel itself, aid the flow of lymph. The same factors are responsible for transporting the fluid throughout the lymphatic network up to the thoracic duct. (Schulte-Merker et al., 2011; Zawieja, 2009)

Collecting vessels are surrounded by contracting smooth muscle cells and are divided into small functional units called lymphangions that are separated by valves. When lymph is transported into collecting vessels, it forms a pressure gradient that opens the valves downstream allowing lymph to flow forward, and simultaneously closes upstream valves preventing the backflow of lymph. In addition, contracting smooth muscle cells ensure the directional flow of lymph along the lymphatic network towards the bloodstream. (Morfoisse and Noel, 2019; Zawieja, 2009)

2.1.3.2 Lipid absorption

Lipids are absorbed by lymphatic capillaries in the small intestine villi i.e. lacteals. In contrast to initial lymphatics in the periphery, lacteals contain button-like and zipper juctions and they are found in close relation to contracting smooth muscle cells of the intestinal lamina propria. Lipid molecules are first absorbed by intestinal epithelial cells, which release them as mature chylomicrons that are actively taken up by lacteals. Chylomicrons are transported from lacteals to the collecting mesenteric lymphatic vessels and further to the systemic circulation. The flow of the intestinal lymphatic vessel content is ensured by contraction of the smooth muscle cells surrounding the lacteals, gut movement and contraction of the collecting mesenteric lymphatic vessels. Other molecules, such as fat-soluble vitamins and gut hormones, also get access to the lymphatics in chylomicrons. Albeit being one of the main functions of the lymphatic system, much remains to be discovered about the underlying mechanisms of lipid absorption and transport by the lymphatics. (Bernier-Latmani and Petrova, 2017; Hokkanen et al., 2019)

2.1.4 The lymphatic system in disease

2.1.4.1 Abnormal fluid homeostasis

The main function of the lymphatic system is to regulate tissue fluid homeostasis, which if impaired, leads to lymphedema. Lymphedema is distinguished by an increase in protein-rich fluid in the tissue leading to the swelling of extremeties, fat accumulation, fibrosis and other symptoms. This largely disabilitating condition can be primary or secondary. Primary lymphedema is a rare, hereditary condition and it is related to defects in lymphatic development as well as impaired valve function. In the Western world, secondary lymphedema is most often a result of radiotherapy or surgery used to treat breast cancer. In tropical countries, secondary lymphedema often results from lymphatic filariasis, a parasitic infection. Lymphedema can only be treated palliatively as no curative treatment exists. (Cueni and Detmar, 2008)

2.1.4.2 Role in cancer

A majority of solid tumors disseminate through the lymphatic system and form metastases in the sentinel or more distant lymph nodes. Lymph node metastases are regarded as a prognostic factor, which is often associated with reduced patient survival. The formation of metastases via the lymphatics is an actively regulated process. It starts with an increase of peritumoral lymphatics followed by cancer cell dissemination into the lymphatic vessels, and finally, the entry of tumor cells into the lymph node. Peritumoral lymphangiogenesis can be initiated by tumor-derived factors that also prepare the sentinel lymph node for metastases. Lymph node LECs have been shown to secrete factors that attract tumor cells into the lymph node. Therapeutic advances in this field are necessary for more effective patient care. (Alitalo and Detmar, 2012; Padera et al., 2016)

2.2 The lymph node

There are several hundreds of lymph nodes in humans that are situated strategically around the body in its periphery, in close vicinity to internal organs and the gut. Lymph nodes are connected by lymphatic vessels and are assembled in chains. Lymphatic vessels leading to the lymph node are called afferent lymphatics and vessels leaving the node are the efferent lymphatic vessels. Lymph nodes are the main organs that filter lymph. Depending on their anatomical location, lymph nodes encounter very different lymph-borne molecules. The main immunological function of the lymph node is to provide the

settings for initiating adaptive immune responses against foreign antigens delivered in lymph or transported within antigen-presenting DCs that migrate to draining lymph nodes. In addition to recognizing foreign antigens, the lymph node harbors cells important for the maintenance of tolerance against the host itself. (Girard et al., 2012)

2.2.1 Lymph node organogenesis

During embryonic development in the mouse, primitive lymph sacs rise at sites of major vascular junctions from E10.5 onward, which then develop into the early lymph node anlagen around E13. The main cells involved in lymph node organogenesis are lymphoid tissue inducer (LTi) cells of hematopoietic origin, and lymphoid tissue organizer (LTo) cells, which derive from mesenchymal cells. These cells interact via signaling of lymphotoxin-β receptor (LTβR) and its ligand lymphotoxin-$\alpha_1\beta_2$ ($LT\alpha_1\beta_2$). Other crucial signaling molecules include interleukin-7 (IL-7) and the RANK-RANKL pathway. (Mebius, 2003; van de Pavert and Mebius, 2010)

LTi cells rise from the fetal liver (Mebius et al., 2001), are the first cells to enter the developing lymph node anlagen (Mebius et al., 1997), and express many molecules essential for lymph node development, such as $LT\alpha_1\beta_2$ (Mebius, 2003). Cytokines, namely receptor activator of nuclear κ B (RANK) and IL-7 can further induce $LT\alpha_1\beta_2$ expression in LTi cells (Yoshida et al., 2002). The early lymph node anlagen also harbors immature stromal cells expressing several adhesion molecules, which mediate the binding of LTi cells to the stroma. This allows LTβR to interact with its ligand resulting in the maturation of stromal cells into functional LTo cells. (Bénézech et al., 2010) As new LTi cells arrive to the anlagen, they upregulate $LT\alpha_1\beta_2$ due to signaling via LTβR, which provides a positive feedback loop regulated by LTo cells. (Cupedo et al., 2004; Vondenhoff et al., 2009)

LTi cell interaction with LTo cells triggers lymphotoxin signaling and leads to clustering of the LTi cells. This is contingent on adhesion molecules expressed by LTo cells, as well as chemokines that attract new LTi cells to the anlagen. The chemokine CXCL13 is important for the formation of lymph nodes, as mice lacking it fail to develop many peripheral lymph nodes. (Ansel et al., 2000) Other chemokines and chemokine receptors, such as CCL19, CCL21, CCR7 and CXCR5, also play a role in lymph node development (Luther et al., 2003; Ohl et al., 2003). Chemokines attract new LTi cells to the lymph node anlagen contributing to the established positive feedback loop between LTi and LTo cells. Neuron-derived factors have been suggested to induce expression of CXCL13 in the mesenchymal stromal cells of the anlagen. Indeed, CXCL13 has a major role

in attracting the first LTi cells to the anlagen, and therefore, factors inducing its expression take part in the early formation of the lymph node. (Van De Pavert et al., 2009)

A long-prevailing concept was that LTo cells derive solely from a mesenchymal origin and regulate lymph node development via LTβR-signaling. However, lymph nodes develop even when LTβR-signaling in LTo cells is abrogated, suggesting that other regulatory cells exist. (Chai et al., 2013) If the LTβR coding gene *Ltbr* is depleted from LECs, the development of a substantial portion of lymph nodes is blocked, suggesting that LECs can also act as LTo cells (Onder et al., 2013). In fact, LECs are able to retain LTi cells in the lymph node anlagen providing the initial step for successful lymph node formation (Onder et al., 2017), as early blood vessels cannot support the CXCL13-induced recruitment of LTi cells to the anlagen (Onder and Ludewig, 2018). LECs are also needed for creating a CCL21 gradient to attract LTi cells (Onder et al., 2017) that can exit venules and enter the developing lymph node (Bovay et al., 2018).

After the lymph node anlagen has developed, the lymph node goes under structural remodeling. The lymphatic and vascular infrastructure expands to facilitate the incoming lymphocytes starting at E18. In addition, the stromal cell compartment develops to support the hierarchical structure of the hematopoietic cells in the lymph node. The compartmentalized lymph node architecture is further defined by several factors resulting in a highly developed, central organ of the immune system. (Onder and Ludewig, 2018; Randall et al., 2008)

2.2.2 Lymph node structure

The lymph node is a compartmentalized organ divided into several structural regions. Surrounded by a fibrous capsule, the lymph node is comprised of the SCS, cortex, paracortex and medullary sinus (Figure 1). The major cells populating the lymph node are immune cells that are involved in recognizing foreign antigens and mounting corresponding immune responses. Stromal cells, including LECs, BECs and fibroblastic reticular cells (FRC) represent a small percentage of the total lymph node cells, yet they are indispensable for the function of the lymph node. Each region of the lymph node harbors a different set of immune and stromal cells with its own distinct functions. (Mueller and Germain, 2009)

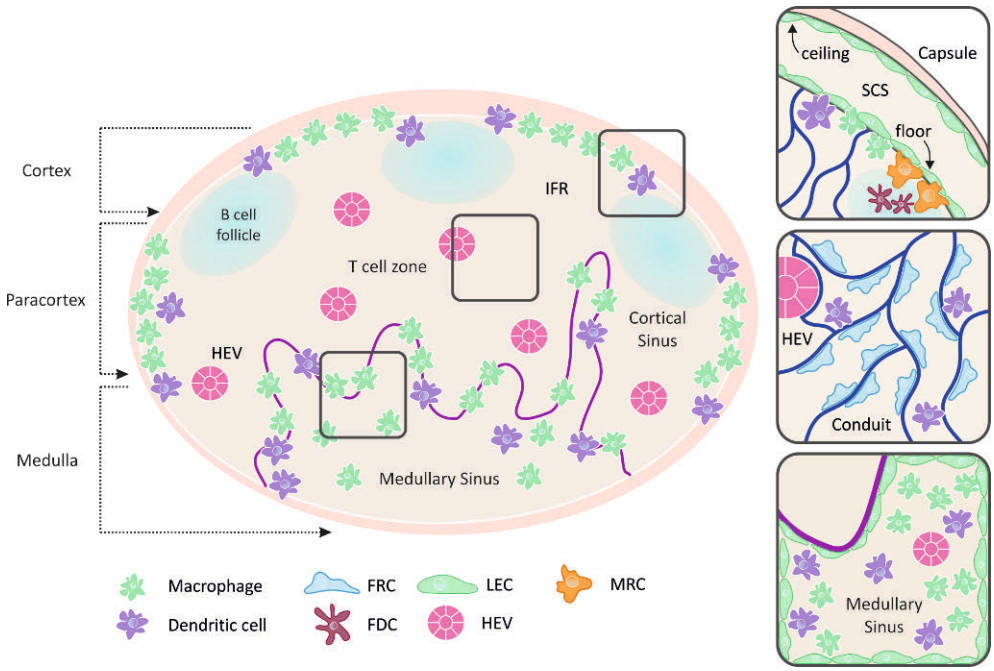

Figure 1. A schematic presentation of the lymph node structure showing the main cellular and structural components related to this thesis. The lymph node can be divided into the cortex, paracortex and the medullary sinus regions. Close-up images highlight the subcapsular sinus (SCS) showing the location of important stromal cells, the conduit network present in T cell zone as well as the medullary sinus. FDC, follicular dendritic cell, FRC fibroblastic reticular cell, HEV high-endothelial venule, IFR, interfollicular region, MRC marginal reticular cell, LEC lymphatic endothelial cell.

2.2.2.1 Lymph node lymphatics

LECs can be found in the SCS, cortical and medullary sinuses of the lymph node. Afferent lymphatic vessels leading to the lymph node penetrate its capsule and open up into the SCS. This hollow space under the capsule is lined by LECs that form the ceiling and floor of the sinus. Unlike LECs in the periphery, lymph node LECs have different expression patterns of lymphatic markers. LECs in the ceiling and floor both express Prox1 and VEGFR-3, but the ceiling can be distinguished by expression of the chemokine CCL1, chemokine receptor CCRL1 and podoplanin, whereas LECs in the SCS floor are positive for LYVE1 and CCL21. (Das et al., 2013; Qu et al., 2004; Ulvmar et al., 2014) Lymph node LECs also express PLVAP in a distinct set of cartwheel-like subcellular structures called diaphragms, where it regulates endothelial permeability (Rantakari et al., 2015). LECs take part in regulating lymphocyte survival by producing several survival factors, such as IL-7 (Malhotra et al., 2012).

In the lymph node, LECs are also present in the cortical sinuses that span the paracortex of the lymph node being the site where lymphocyte egress is initiated. Cortical sinuses attach to the medullary sinus leading to the efferent lymphatic vessels, the final exit route for lymphocytes. (Girard et al., 2012) The medullary sinus harbors medullary cords, where plasmablasts migrate to mature during humoral immune responses (Luther et al., 1997). These cords contain a blood vessel and are surrounded by LECs that separate the medullary cord content and blood vessel from the medullary sinus (Huang et al., 2018; Pfeiffer et al., 2008).

The heterogeneity of lymph node LECs has recently gained interest, as techniques to study it have become availabe. Using microscopic analyses, LECs of the human lymph node were first divided into two subpopulations based on expression of LYVE1 and Stabilin-2 in the paracortical and medullary sinuses, and their absence in the SCS (Park et al., 2014). Using single-cell RNA sequencing, a more sophisticated and informative technique, Takeda and coworkers identified six different populations of LECs in the human lymph node. This suggests that LECs have distinct functions related to their location in the lymph node, which remain yet to be fully described. (Takeda et al., 2019)

2.2.2.2 Lymph node blood vasculature

The lymph node blood vasculature consists of arterioles, capillaries, unique post-capillary venules termed high endothelial venules (HEV) and venules, all lined by BECs. Blood vessels enter and exit the lymph node at the hilus area near the efferent vessels, and they circulate throughout the whole lymph node. Capillaries provide oxygen and nutrients to the lymph node and convey migrating immune cells that exit the blood vasculature from HEVs. (Girard et al., 2012; Kowala and Schoefl, 1986) Endothelial cells of capillaries and HEVs differ significantly in their gene expression reflecting their clear functional differences. Whereas capillary endothelial cells express genes related to vascular development, endothelial differentiation and fluid transport, HEV-expressed genes are related to lymphocyte migration, inflammatory response and lymph node development. (Lee et al., 2014) HEVs have a distinct morphology that is not found in other venules. Their endothelial cells are plump and cuboidal, the venules are surrounded by a thin basal lamina further ensheathed by FRCs with collagen-containing bundles, and they are wrapped by surrounding pericytes. (Anderson et al., 1976; Freemont and Jones, 1983)

HEVs are the prominent exit site for lymphocytes entering the lymph node parenchyma. They also harbor lymphocytes waiting for entry in ’pockets’ between the endothelial cells and the surrounding stroma. The constant presence of lymphocytes contributes to the morphology of HEVs, as in the absence of

lymphocytes they become flattened. (Mionnet et al., 2011) Lymph node HEVs are best distinguished from other vascular endothelial cells by their expression of the MECA-79 antibody epitope, peripheral node addressins (PNAd), which are vital for lymphocyte migration (Streeter et al., 1988). In addition, HEVs express several other molecules involved in lymphocyte migration that are further discussed in section 2.3.2.

Single-cell sequencing reveals endothelial cells in HEVs to be a heterogeneous cell population exhibiting spatial differences in the expression of molecules important for lymphocyte migration (Veerman et al., 2019). Interestingly, DCs regulate the HEV phenotype by maintaining their mature phenotype. If DCs are depleted from the lymph node, HEVs dedifferentiate into an immature state, which reduces lymphocyte migration into the lymph node. DCs express the ligand for HEV-expressed LTβR, and this signaling is required for the mature phenotype of HEVs and successful lymphocyte migration. (Moussion and Girard, 2011) In addition to DCs, FRCs also regulate HEVs via LTβR regulated expression of VEGF, which is needed for the proliferation and maintenance of the blood vasculature (Chyou et al., 2008).

2.2.2.3 Reticular cells of the lymph node

A major cell population of the lymph node stromal cell compartment is the mesenchymal cell derived reticular cell population. These cells are characterized as podoplanin$^+$ and are negative for CD31 and CD45 separating them from endothelial and hematopoietic cells, respectively. A majority of reticular cells are FRCs, but the population also contains marginal reticular cells (MRC) found in the SCS, follicular dendritic cells (FDC) supporting B cell follicles and other specialized podoplanin$^+$ stromal cells. Stromal cells also contain a 'double negative' population negative for both podoplanin and CD31. (Malhotra et al., 2013)

2.2.2.3.1 Fibroblastic reticular cells

FRCs produce a reticular network that spans throughout the lymph node and is most prominent in the T cell zone of the paracortex. The reticular network, also termed the conduit system, is ensheathed by FRCs that produce the ECM of the conduit. The conduit core consists of collagen I fibres that are surrounded by an ER-TR7$^+$ microfibrillar zone, a basement membrane and finally the FRC itself. (Kaldjian et al., 2001; Sixt et al., 2005) The reticular network offers structural support and elasticity for the lymph node and regulates the spatial regionality of lymphocytes by guiding their migration. Moreover, the conduit network regulates

the size-selective entry of antigens into the lymph node contributing to the lymph node filtering function. (Mueller and Germain, 2009)

FRCs span the whole lymph node from the SCS to the medullary sinus. Based on single-cell sequencing, FRCs can be divided into nine distinct populations located in different regions of the lymph node. In addition to the T cell zone, independent FRC populations were identified in the interfollicular (IFR) regions of the cortex, at the interface of follicles and the T cell zone, in the perivascular region, and finally in the medullary sinus. (Rodda et al., 2018) Transcriptional analyses define the 'double negative' population of FRCs as contractile, pericyte-like cells located around vessels and in the medullary sinus (Malhotra et al., 2012).

FRCs regulate the intranodal migration of newly entering lymphocytes by expressing CCL19 and CCL21, ligands of CCR7 (Bajénoff et al., 2006a). FRCs also support naïve T cell survival by expressing IL-7 and CCL19 (Link et al., 2007) in addition to supporting B cell survival by secreting the B cell activating factor, BAFF. When FRCs are depleted from the lymph node, its cortex and B cell follicles lose their structural integrity and B cells fail to conduct proper humoral responses. (Cremasco et al., 2014) Moreover, specialized medullary reticular cells present in medullary cords are important for the regulation of plasmacell homeostasis through the secretion of survival factors (Huang et al., 2018).

2.2.2.3.2 Follicular stromal cells

The main stromal cells present in the lymph node B cell follicles are FDCs, which secrete CXCL13 attracting and retaining B cells in the follicles (Cyster et al., 2000). Depleting FDCs disrupts the separation of the T cell zone and B cell follicles (Wang et al., 2011). When B cells go under activation, germinal centers (GC) arise within follicles. GCs can be divided into dark and light zones based on the supporting stromal cells. FDCs are present in the light zone, whereas CXCL12-secreting reticular cells colonize the dark zone that contains proliferating B cells. (Rodda et al., 2015)

MRCs are a distinct stromal cell population present in the SCS at the top of B cell follicles. They can be distinguished from other podoplanin^{+} stromal cells by expression of mucosal vascular addressin cell adhesion molecule 1 (MAdCAM-1) and RANKL. (Katakai et al., 2008) Similar to FDCs, MRCs secrete CXCL13 attracting B cells to follicles, where they can also present antigens to B cells (Katakai, 2012). MRCs are also known to create a niche supporting innate lymphoid cells in the SCS (Hoorweg et al., 2015).

2.2.2.4 Lymph node immune cells

2.2.2.4.1 Lymphocytes

Immune cells mainly reside in the cortex and paracortex areas of the lymph node. Naïve B cells form follicles in the cortex, where B cell responses take place. Upon antigen recognition, B cells are activated and can differentiate into plasmablasts, which are able to rapidly produce antibodies. Alternatively, B cells enter GCs within the follicles containing mitotic B cells. In GCs, B cells undergo affinity maturation resulting in their differentiation into specific antibody secreting plasma cells, some of which stay in the lymph node whereas others migrate to the bone marrow. (Batista and Harwood, 2009) Antigens are presented to B cells by several different cell types discussed later. Naïve B cells enter the lymph node via HEVs in the paracortex and migrate to the cortex following a CXCL13 chemokine gradient secreted by FDCs. (Girard et al., 2012) A low number of NK cells, which develop from the same progenitors as lymphocytes, are present in the lymph node during steady state. They reside at the border between follicles and T cells as well as in the medullary sinus. (Bajénoff et al., 2006b; Garrod et al., 2007)

$CD4^+$ and $CD8^+$ T cells are retained in the paracortex by chemokine signals that guide their entry and intranodal migration. They express the chemokine receptor CCR7, which responds to its ligands CCL19 and CCL21 expressed by FRCs. Antigen-presenting DCs also reside in the paracortex, which increases the likeliness of a naïve T cell to encounter its cognate antigen presented by a DC. (Girard et al., 2012) After encountering antigens, $CD4^+$ T cells mature into effector cells of different subtypes such as Th1, Th2, Th17, T follicular helper cells (Tfh) and induced T regulatory cells (Treg), depending on the activating stimuli. These cells migrate into different regions of the lymph node in a CXCR3-dependent manner, where antigen encounter is highly probable, such as the IFR of the cortex and the medullary sinus. IFRs receive a large load of antigens within DCs migrating from the periphery or directly from the SCS, and they are sites for direct priming of T cells in addition to harboring memory cells. (Groom, 2019; Groom et al., 2012)

2.2.2.4.2 Resident macrophages

Lymph node macrophages are mainly located within its sinuses, where they are in direct contact with the antigen-rich lymph. These macrophages can be divided into four populations: subcapsular sinus macrophages (SSM, $CD169^+$ $F4/80^–$), medullary sinus macrophages (MSM, $CD169^+$ $F4/80^+$), medullary cord macrophages (MCM, $CD169^–$ $F4/80^+$) and paracortical macrophages ($CD68^+$) that

possibly overlap with T cell zone marcophages (CX3CR1^{hi} CD64^{+} MERTK^{+}). (Baratin et al., 2017; Gasteiger et al., 2016) SSM line the SCS floor by sitting beneath the endothelial cell layer. These macrophages extend a part of their cell body into the SCS lumen while simultaneously reaching into B cell follicles with long cellular protrusions. This creates a web of several different cell types all intermingled in the SCS floor. SSM are poorly endocytic but they retain immunocomplexes on their surface for antigen presentation to B cells. (Phan et al., 2007, 2009) MSM reside in the lymph node medulla and in regions between B cell follicles and the medullary sinus, where these highly phagocytic cells trap lymph-borne antigens and take part in their clearance (Grigorova et al., 2009). T cell zone macrophages are located in the T cell region where they dispose of apoptotic cells (Baratin et al., 2017).

The SCS is structurally intriguing as it harbors several different cell types that create a suitable niche for SSM survival. RANK is expressed by both MRC and LECs in the SCS, which is required for the survival for SSM and MSM. Moreover, LECs in the SCS and medullary sinus are the major cell type secreting colony stimulating factor 1 (CSF1), the master regulator of macrophage survival, which contributes to the maintenance of lymph node sinus macrophages. (Camara et al., 2019; Mondor et al., 2019)

2.2.2.4.3 Dendritic cells

DCs are professional antigen presenting cells that are essential for the initiation of adaptive immune responses. In homeostatic conditions, DCs maintain tolerance by introducing self-molecules to T cells, whereas they transport antigens from the periphery to secondary lymphoid organs during inflammation. The lymph node harbors several types of DCs, both resident i.e. classical DCs (cDC) and migratory cells. At steady state, dermal DCs and Langerhans cells migrate from the skin to the lymph node via the lymphatics. Their migration is greatly increased during inflammation, when they actively present antigens to naïve T cells in the lymph node. The lymph node is largely populated by resident DCs that arrive via the blood vasculature. Resident DCs can be roughly divided into CD8α^{+} CD11b^{-} cDC1 or CD8α^{-} CD11b^{+} cDC2 populations and plasmacytoid DCs. (Itano and Jenkins, 2003; Randolph et al., 2008; Worbs et al., 2017) The deep T cell zone is predominantly occupied by cDC1 cells, which present antigens more efficiently to CD8^{+} T cells. In contrast, cDC2 cells populate areas proximal to lymphatic sinuses, where they activate CD4^{+} T cells. After migratory DCs arrive to the lymph node, they populate the lymph node in a mixed pattern. The regionality of resident DCs is reflected by the T cell proliferation they induce; as T cells proliferate in the locations they have been activated in. (Gerner et al., 2012, 2017)

2.2.3 Function of the lymph node

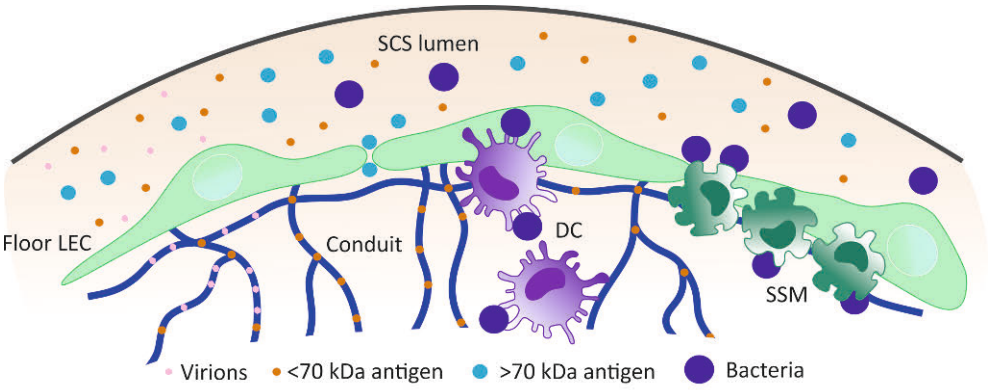

Figure 2. Antigens arriving to the lymph node are sorted based on their size allowing <70 kDa molecules to directly enter the conduit system. Large soluble antigens (>70 kDa) are excluded from the lymph node parenchyma, yet some are able to diffuse through gaps in the subcapsular sinus (SCS) floor over time. Resident subcapsular sinus macrophages (SSM) and dendritic cells (DC) phagocytose bacteria from the SCS and transport large soluble antigens to B cells, whereas virions are able to enter the conduit. LEC, lymphatic endothelial cell.

2.2.3.1 Transporting antigens

2.2.3.1.1 Role of the reticular conduit

Lymph delivers antigens from the periphery to the lymph node, where they are sorted in the SCS based on their size. The conduit instantly delivers small soluble antigens with a size under 70 kDa from the SCS to the lymph node parenchyma and the luminal surface of HEVs. The size limit is a feature of the collagen core of the conduit, which physically only allows molecules with a hydrodynamic radius below 5-6 nm (equivalent to molecular weight <70 kDa for globular proteins) to enter the conduit (Figure 2). Large lymph-borne molecules, such as antibodies (size 150 kDa) do not get access to the parenchyma and are instead concentrated in the SCS and medullary sinus, where they slowly begin to penetrate into the parenchyma. (Gerner et al., 2017; Gretz et al., 2000; Roozendaal et al., 2009; Sixt et al., 2005) The sorting of lymph-borne molecules in the SCS is orchestrated by PLVAP – a protein sieve in LECs that sits on top of the conduit openings performing size-selective sorting. In mice deficient of PLVAP, large antigens up to 500 kDa have access to the conduit, which leads to increased uptake of antigens by macrophages and DCs in the lymph node parenchyma. (Rantakari et al., 2015)

The conduit system also transports fluids and endogenous soluble molecules in addition to foreign antigens. Large volumes of fluids flow within distinct conduits associated with the lymph node capsule that are comprised of elastin fibers (Lin et al., 2018). Chemokines are produced in the periphery during inflammation and are collected into draining lymph nodes. Here, chemokines enter the conduit and are delivered to the bloodstream through HEVs to recruit lymphocytes into the lymph node. (Gretz et al., 2000) Locally produced IgM in lymph nodes is taken up by the conduit and exported out of lymph nodes through the vasculature during immune responses. As pentameric IgM is a large molecule (size 900 kDa), it seems that the size-selective nature of the conduit is related to the SCS and in fact, large molecules are able to gain access to the conduit when already present in the lymph node parenchyma. (Thierry et al., 2018)

2.2.3.1.2 Large antigens

In the periphery, DCs phagocytose large antigens, such as microbes, and migrate into draining lymph nodes to present microbe-derived peptides to naïve T cells. Migratory DCs arrive to the lymph node in approximately 12 hours after inflammatory stimuli. However, some studies have shown DCs to ingest antigens in the draining lymph nodes before the arrival of migratory DCs. (Itano and Jenkins, 2003) FRCs cover about 90% of the conduit network and 60-80% of lymph node resident DCs are in direct contact with this network. These resident cells are able to sample and process soluble antigens directly from the conduit, and initiate immune responses before migratory DCs get access to the lymph node. (Sixt et al., 2005)

Immunocomplexes, i.e. molecular complexes formed by the binding of antibodies to antigens, are taken up directly from the lymph by SSM in the SCS lumen. Follicular B cells capture these immunocomplexes from the elongated protrusions of the macrophages and deliver the antigens to FDCs in a complement receptor dependent manner. (Phan et al., 2007) Moreover, large experimental peptide:MHCII (major histocompatibility complex class II) complexes can slowly diffuse directly from the SCS to the interstitium surrounding parenchymal lymphocytes several minutes after being subcutaneously (s.c.) administered. Diffusion is thought to take place through small gaps in the SCS floor and it is independent of any receptor or the conduit system. (Gerner et al., 2017; Pape et al., 2007; Roozendaal et al., 2009) In contrast, small immunocomplexes resemble small soluble antigens and get access to the lymph node by entering the reticular conduit present in B cell follicles (Roozendaal et al., 2009).

2.2.3.1.3 Infectious agents

Afferent lymphatics deliver pathogens from the periphery to draining lymph nodes, where different cells actively combat infection primarily in the lymph node sinuses. Several viruses can infect the poorly phagocytic SSM, which is critical in preventing systemic dissemination of the pathogen. These macrophages transfer viruses from the SCS to follicular B cells to initiate humoral immune responses. (Junt et al., 2007; Kastenmüller et al., 2012) In addition to macrophages, the SCS harbors a specialized population of resident DCs that also phagocytose bacteria from the SCS lumen and induce T cell activation (Gerner et al., 2015). Resident DCs in the lymph node medulla are also involved in the internalization of viruses, which subsequently induces DC migration towards FDCs followed by the initiation of a humoral immune response (Gonzalez et al., 2010). Surprisingly, despite being large, viruses are also able to enter the reticular conduit upon infection and infect paracortical DCs followed by T cell activation. The ability of viruses to enter the conduit is regulated by PLVAP, as its absence markedly increases the viral load of the conduits as well as the number of infected cells. (Reynoso et al., 2019)

2.2.3.2 Stromal cells regulate peripheral tolerance

Several lymph node stromal cells are able to present antigens and have a role in maintaining peripheral tolerance. Although DCs are the professional antigen presenting cells, stromal cells are also able to directly present antigens to T cells and induce their proliferation. However, as stromal cells lack costimulatory molecules, proliferating T cells are unable to excert effector functions and instead, they undergo deletion. (Hirosue and Dubrot, 2015) Lymph contains many self-antigens, as it is concentrated with the proteins and peptides derived from the tissues it drains. Therefore, self-antigens are readily available for stromal cells in the lymph node, especially LECs that are directly in contact with the lymph in the SCS. (Clement et al., 2011)

Peptides derived from intracellular antigens are presented on MHCI (major histocompatibility complex class I) molecules to cytotoxic $CD8^+$ T cells. Stromal cells (FRC, LEC, BEC) express several peripheral tissue antigens often in distinct subpopulations, which they can present to $CD8^+$ T cells leading to tolerance against these self-antigens. (Cohen et al., 2010; Fletcher et al., 2010; Hirosue and Dubrot, 2015) LECs are also able to directly scavenge and internalize foreign antigens present in lymph and present them to $CD8^+$ T cells that undergo activation. In contrast to DCs, LECs express high levels of the inhibitory molecule PD-L1 and cause upregulation of PD-1 and CTLA4 on lymphocytes. This dampens the activation of T cells resulting in the deletion of antigen specific T cells and maintenance of tolerance. Antigen presentation conducted by LECs is therefore

significant in regulating downstream effects and has a role in preventing autoimmunity. (Hirosue et al., 2014; Tewalt et al., 2012)

In addition to expressing MHCI, lymph node stromal cells (FRC, LEC, BEC) express MHCII that is required for the presentation of extracellular antigens to $CD4^+$ T cells (Malhotra et al., 2013). LECs are able to synthesize MHCII, or they can acquire it directly from DCs. Yet, LECs are unable to initiate $CD4^+$ cell proliferation as they lack molecules of the antigen presentation machinery. However, DCs may acquire tissue antigen-derived peptides from LECs, present them to T cells and induce T cell anergy in the lymph node. Thereby, LECs act as a reservoir for tissue antigens and they can indirectly regulate T cell tolerance. (Rouhani et al., 2015) LECs and FRCs can also acquire peptide:MHCII complexes from DCs by direct cell-cell contact or within exosomes. In such case, they are able to directly promote $CD4^+$ T cell apoptosis. (Dubrot et al., 2014) Stromal cells also maintain regulatory T cells in lymph nodes by presenting antigens directly to them via MHCII (Baptista et al., 2014).

2.3 Immune cell migration

Immune cells migrate into lymph nodes via two different routes: the afferent lymphatics and the blood circulation. The main cell populations migrating via the afferent lymphatics are $CD4^+$ effector and memory T cells as well as DCs under homeostatic conditions (90% and 10% of afferent lymph cell composition, respectively). However, a low number of B cells, monocytes and granulocytes can also be detected in the afferent lymph. Naïve T cells mainly enter lymph nodes via HEVs of the blood vasculature in search of foreign antigens. If naïve T cells do not encounter their cognate antigens after 6-12 hours of scanning DCs in the lymph node, they will egress the lymph node via the efferent lymphatics. Subsequently, the cellular composition of lymph changes drastically after it flows through the lymph node resulting in efferent lymph being mainly composed of naïve lymphocytes that originally entered the lymph node through HEVs. (Hall and Morris, 1962; Hunter et al., 2016; Mackay et al., 1988; Smith et al., 1970) A schematic overview of immune cell migration described in detail hereafter is presented in Figure 3. Immune cell recirculation between lymph nodes and the blood circulation occurs in peaks, where cell entry into lymph nodes peaks at the onset of active time and egress is most active during the resting phase. The circadian rhythm of immune cell migration is controlled by clock-genes expressed in lymphocytes, which regulate the molecules that are mainly involved in migration. Adrenergic nerves can also regulate the circadian rhythm. In both cases, oscillations in cell migration affect adoptive immunity. (Druzd et al., 2017; Nakai et al., 2014)

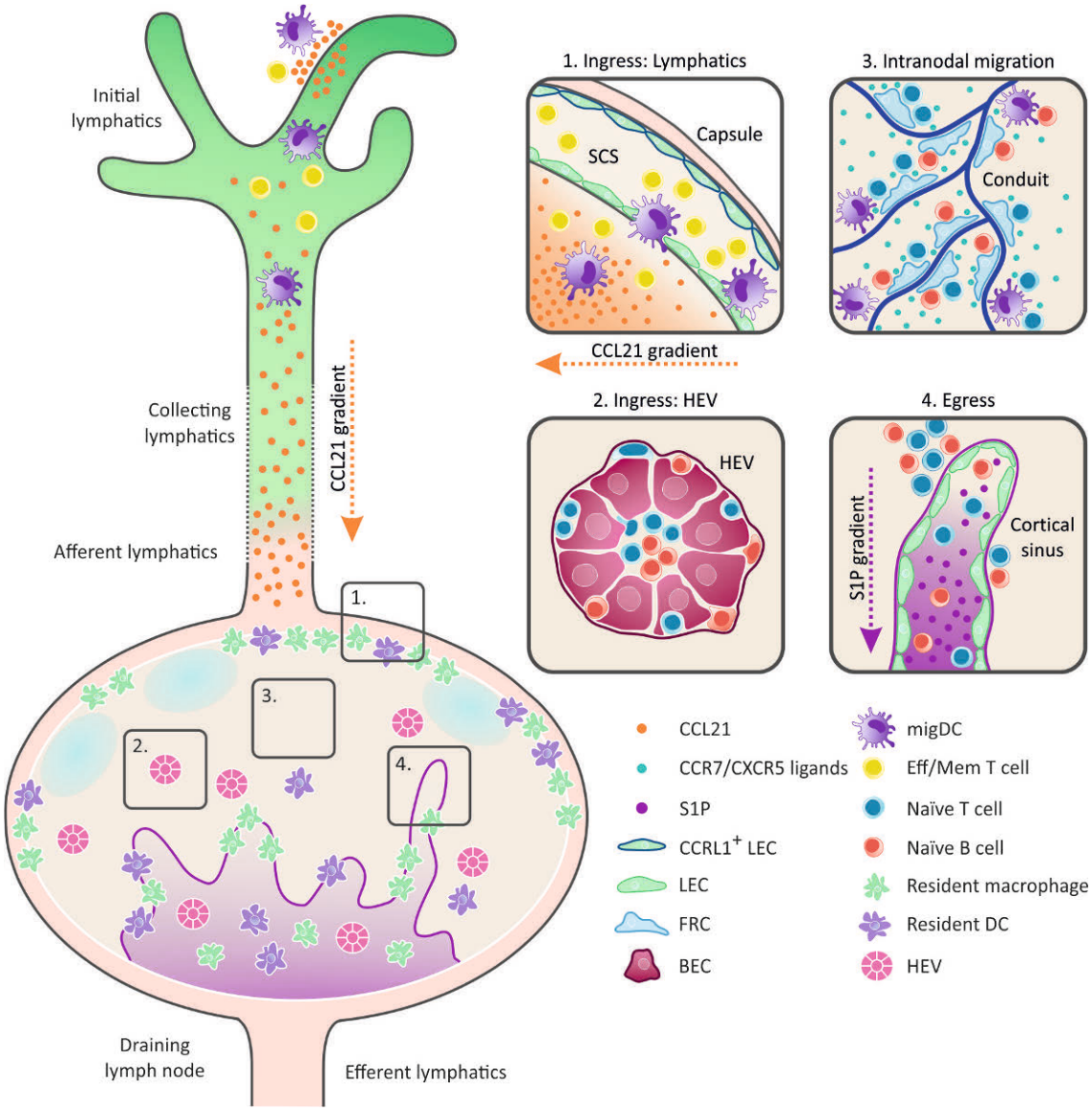

Figure 3. A schematic view of immune cell migration into lymph nodes highlighting the most important molecular gradients regulating cell migration. Migratory dendritic cells (migDC) as well as effector and memory T cells (Eff/Mem T cell) are able to enter the initial lymphatics following local CCL21 gradients and migrate to the draining lymph node. Immune cells arriving among the lymph cross the subcapsular sinus (SCS) to enter the lymph node parenchyma following a CCL21 gradient, which is regulated by CCRL1-expressing lymphatic endothelial cells (LEC, 1). Naïve T and B lymphocytes migrate to lymph nodes in the bloodstream and enter the node via specialized high-endothelial venules (HEV, 2). Lymphocytes migrate intranodally to their allocated locations guided by CCR7/CXCR5 and their ligands, which are produced by fibroblastic reticular cells (FRC, 3). Lymphocyte egress takes place at the cortical sinus, where lymphocytes follow a sphingosine-1-phosphate (S1P) gradient into the sinus and to the efferent lymphatics thereafter (4).

2.3.1 Migration via afferent lymphatics

2.3.1.1 Intravasation into lymphatics

Immune cells enter afferent lymphatics in peripheral tissues and migrate to the draining lymph node. The main molecules regulating DC migration via the afferent lymphatics are the chemokine receptor CCR7 expressed by DCs themselves, and its ligand, CCL21. DCs migrate towards the inital blunt-ended lymphatics attracted by a CCL21 gradient that is formed by the constant secretion of CCL21 by LECs. (Weber et al., 2013) Once DCs come into contact with the lymphatic vessel, they attach onto its abluminal side and cluster around pores of the basement membrane, where they actively push the basement membrane aside creating space for entry. (Pflicke and Sixt, 2009; Tal et al., 2011) DCs probe the abluminal surface of LECs with cellular extensions causing them to release a burst of CCL21 that accumulates at the endothelial cell junctions. DCs then migrate into the lymphatic vessel lumen through the flaps between endothelial cell tight junctions guided by the high local concentration of CCL21. (Vaahtomeri et al., 2017) In the vessel lumen, DCs crawl downstream along the lymphatic capillaries directed by an intraluminal CCL21 gradient. Finally, DCs enter collecting lymphatic vessels, where they are passively transported to the draining lymph node by lymphatic flow. (Russo et al., 2016)

Similar to DCs, T cell migration into skin lymphatics and the draining lymph node is dependent on CCR7 under steady state and acute inflammation. Although being a minor cell population in the afferent lymph, naïve T cells have also been observed in parenchymal tissues such as the skin and they are able to migrate into draining lymph nodes under experimental settings. (Cose et al., 2006; Debes et al., 2005) During chronic inflammation, T cell intravasation into the initial lymphatics is CCR7-independent and is regulated by the ICAM-1/LFA-1 pathway instead. After entering the initial lymphatics, T cells actively crawl along the endothelium by adhering to LEC-expressed ICAM-1 (Intercellular Adhesion Molecule 1) until they reach the collecting lymphatics, where cells are transported passively to the draining lymph node among lymph. (Brown et al., 2010; Teijeira et al., 2017) While crawling in the lymphatics, T cells may interact with DCs allowing for T cell priming to begin already in the periphery (Hunter et al., 2019). Although integrins do not play a role in DC migration under steady state (Lämmermann et al., 2008), they regulate DC migration during chronic inflammation (Johnson et al., 2006).

Podoplanin and LYVE1 both have a role in regulating DC migration into the afferent lymphatics and draining lymph node. DCs express CLEC-2 (C-type lectin-like receptor 2), the receptor for podoplanin, and their interaction is required for DCs to get access to the inital lymphatics (Acton et al., 2012). Similarly, the

binding of LYVE1 (expressed by the lymphatics) to its ligand hyaluronan (expressed on DCs) is important for DC adhesion to the lymphatics and their transmigration into the vessel (Johnson et al., 2017). Interestingly, the main regulators of lymphocyte egress from the lymph node, namely sphingosine-1-phosphate (S1P) and its receptor $S1P_1$ also play a role in regulating lymphocyte egress from peripheral tissues. The S1P–$S1P_1$ pathway is mainly involved in the transmigration of T cells through the LEC layer causing retention of T cells in the tissue. (Ledgerwood et al., 2008; Xiong et al., 2019) Moreover, macrophage mannose receptor (CD206) and its ligand CD44 (Salmi et al., 2013), Clever-1 (Karikoski et al., 2009) and CD73 (Ålgars et al., 2011) regulate lymphocyte migration to the draining lymph node via the afferent lymphatics.

2.3.1.2 Migration through the SCS

When arriving to the SCS lumen, cells in the afferent lymph need to cross the barrier of the SCS floor to gain access to the lymph node parenchyma. DCs migrate across the SCS floor and enter the lymph node at IFRs. DC migration is regulated by CCR7 and gradients of its ligand, CCL21 produced by FRCs in the parenchyma. These gradients are controlled by CCRL1, the atypical chemokine receptor expressed by LECs in the SCS ceiling. CCRL1 maintains a gradient by scavenging CCL21 at the opposing position from CCRL1 itself. As a result, the CCL21 gradient is at its lowest right below the SCS floor and increases towards the center of the lymph node. Attracted towards the high concentrations of CCL21 further away in the lymph node parenchyma, DCs migrate through the SCS floor in a CCR7 dependent manner. (Braun et al., 2011; Ulvmar et al., 2014) Lymph-borne monocyte derived DCs use CCR8 to migrate into the draining lymph node. Its ligand, CCL1 is expressed by LECs of the SCS floor, where it regulates the entry of DCs into the parenchyma. (Qu et al., 2004)

T cells injected directly into the afferent lymphatics flow passively to the medullary sinus and enter the lymph node from there. Lymphocytes first migrate into medullary cords, and thereafter cross the $LYVE1^+$ LECs to enter the lymph node parenchyma. However, if T cells and DCs are administered at the same time, T cells will follow DCs in crossing the SCS floor at IFRs. (Braun et al., 2011) Similarly, activated T cells cross the SCS when entering the lymph node parenchyma. Crossing of the SCS is mediated by CCR7 as well as integrins. (Martens et al., 2020) Unlike DCs, T cells can be found in downstream lymph nodes suggesting that many of them pass through the chain of lymph nodes without even entering the lymph nodes (Braun et al., 2011). PLVAP, expressed by the SCS floor LECs, also regulates the entry of lymph-borne lymphocytes into the lymph node. If PLVAP is depleted, lymphocyte transmigration through the SCS is

increased. As PLVAP forms a physical sieve-like structure in LECs, its absence might allow lymphocytes to protrude the LEC layer more readily at points of low resistance leading to increased entry of lymphocytes to the lymph node. (Rantakari et al., 2015) Blocking PLVAP with an antibody results in diminished migration of lymphocytes into the lymph node, a discovery currently challenged by others (Martens et al., 2020; Rantakari et al., 2015).

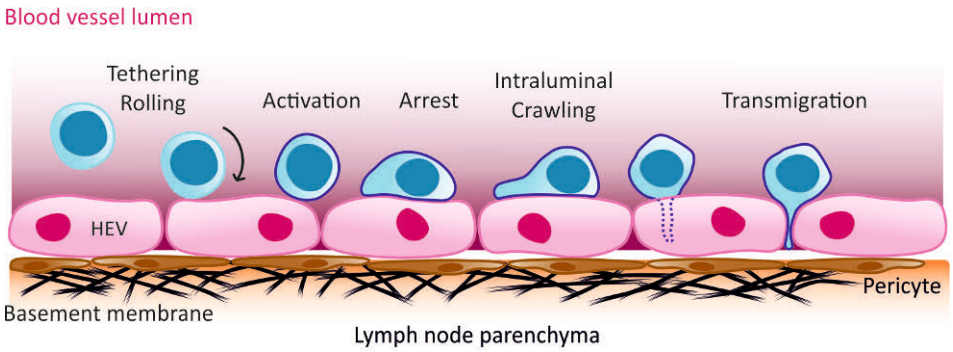

Figure 4. A schematic representation of the homing of lymphocytes to the lymph node via high endothelial venules (HEV). Steps of the adhesion cascade, i.e. the tethering and rolling of lymphocytes, chemokine mediated activation, integrin mediated arrest, intraluminal crawling and transmigration have been illustrated. Figure adapted from Ley *et al.,* 2007.

2.3.2 Migration via the blood vasculature

Naïve B and T cells are the most frequent cells entering the lymph node through the blood vasculature at specialized post-capillary venules, HEVs. During homeostatic conditions, other immune cells such as plasmacytoid DCs, precursory DCs and NK cells are also able to enter the lymph node via HEVs. Lymphocyte migration, often called 'homing', to the lymph node occurs in a multistep adhesion cascade (Figure 4), which involves rolling, activation, adhesion, intraluminal crawling and transmigration of lymphocytes through HEVs. (von Andrian and Mempel, 2003; Butcher and Picker, 1996; Girard et al., 2012)

The first contacts between naïve lymphocytes and the vascular endothelium take place under high blood flow. This tethering, which turns into lymphocytes rolling on the endothelium, is mediated by L-selectin (CD62L) expressed by lymphocytes and its ligand PNAd on HEVs. If blocked by antibodies against L-selectin or PNAd (using the MECA79 antibody) lymphocyte adhesion to the endothelium and subsequent migration into lymph nodes is abrogated. (Berg et al., 1991; Gallatin et al., 1983; Michie et al., 1993; Streeter et al., 1988) PNAd is comprised of several sialomucins (GlyCAM-1, CD34, endomucin, podogalyxin,

endoglycan, nepmucin) that are all recognized by MECA79. L-selectin binds to a specific carbohydrate determinant, namely 6-sulpho sialyl Lewis X present on PNAd and all of its other ligands. (Girard et al., 2012; Rosen, 2004)

The rolling of lymphocytes on the endothelium slows them down and allows them to interact with HEV-bound chemokines to induce integrin activation. HEVs directly express CCL21 bound on heparan sulfate, which interacts with CCR7 expressed on naïve lymphocytes and induces activation of LFA-1 (Lymphocyte Function-associated Antigen 1). (Bao et al., 2010; Gunn et al., 1998; Stein et al., 2000; Warnock et al., 1998) In addition to CCL21, CCL19 and other chemokines can be transcytosed to the HEV lumen, where they can interact with the rolling lymphocytes (Baekkevold et al., 2001). Although CCR7 is the most prominent chemokine receptor involved in integrin activation, CXCR4 also plays a role in the activation of B cell integrins by binding to CXCL12 present on the lumen of HEVs (Okada et al., 2002).

Activated LFA-1 on naïve lymphocytes binds tightly to ICAM-1 expressed on HEVs, which leads to the firm arrest of lymphocytes to the endothelium. By functionally blocking LFA-1, or in mice devoid of LFA-1, the interactions of lymphocytes with the endothelium are inhibited and lymphocyte migration to lymph nodes is dramatically impaired. (Andrew et al., 1998; Hamann et al., 1988) Interestingly, during the rolling of lymphocytes, their deceleration is not because of gradual activation of LFA-1 and subsequent binding of lymphocytes to the endothelium. Instead, LFA-1 activation requires triggering by immobilized chemokines. All of this takes place in seconds and results in an abrupt stop of the rolling lymphocyte at its site of extravasation. (Shamri et al., 2005) ICAM-1 plays a predominant role in lymphocyte arrest to the vascular endothelium, but to a lesser extent, ICAM-2 is involved in the adhesion of lymphocytes that do not adhere to ICAM-1. A small role has been discovered for α4 integrins, namely LPAM and VLA-4 (Very Late Antigen-4) on lymphocytes, and their ligand VCAM-1 (Vascular Cell Adhesion Molecule 1) expressed on the luminal endothelium, in the migration of lymphocytes to lymph nodes. By acting together with LFA-1, α4 integrins are able to regulate lymphocyte entry to lymph nodes in steady state. (Berlin-Rufenach et al., 1999; Boscacci et al., 2010)

A majority of arrested lymphocytes extravasates the HEV at the site they attach to, but roughly a third of lymphocytes crawl along the endothelium before initiating transmigration across HEVs. Crawling can occur in the direction, against it or perpendicular to the blood flow for about 20 μm before cells initiate transmigration. (Boscacci et al., 2010) This type of migration is regulated by the LFA-1/ICAM-1 pathway, while α4 integrins play no role (Park et al., 2010; Shulman et al., 2009). For lymphocytes to successfully transmigrate through the endothelium, they initiate active migration while the endothelial cell undergoes

molecular changes. Lymphocytes respond to exit cues provided by chemotactic and haptotactic gradients created by the endothelial cells, ECM and other immune cells surrounding the blood vessel. The clustering of ICAM-1 and recruitment of VCAM-1 to the site of extravasation is also induced by lymphocyte arrest on the endothelium. Once the transmigrating cell has reached the endothelial cell junction, molecules regulating these junctions, such as VE-cadherin (vascular endothelial cadherin), are relocated to allow the migration of lymphocytes. Other junctional molecules such as the JAM (Junctional Adhesion Molecules) family and CD31 are also involved in lymphocyte transmigration. Most cell transmigration takes place between adjacent endothelial cells (paracellular) but in some cases, lymphocytes migrate through the endothelial cell body (transcellular migration). (Muller, 2016; Nourshargh and Alon, 2014)

The adhesion cascade is well studied and includes a large number of molecules that take part in regulating lymphocyte migration in addition to the ones mentioned. Other selectins, integrins as well as ectoenzymes, such as CD73 and VAP-1 (Vascular Adhesion Protein 1), also play a role in lymphocyte migration. Moreover, during inflammation even more molecules are upregulated, which take part in the homing of neutrophils and other leukocytes. (Ley et al., 2007; Salmi and Jalkanen, 2014) After transmigrating through the HEV endothelial cells, lymphocytes still need to migrate through the surrounding ECM before reaching the lymph node parenchyma. Migration through the basement membrane surrounding HEVs is regulated by autotaxin, a secretory protein expressed by HEVs that is involved in the production of lysophosphatidic acid (LPA). When the autotaxin/LPA pathway is perturbed, lymphocytes are able to cross the endothelial cells of HEVs but they are retained between the endothelium and the basement membrane. (Bai et al., 2013; Hata et al., 2016) Once lymphocytes enter the lymph node parenchyma, their migration is regulated by other factors.

2.3.3 Intranodal migration

Naïve lymphocytes that have migrated via HEVs need to migrate into their designated areas in the lymph node parenchyma. When exiting the vasculature, T cells migrate along the complex stromal network of FRCs partly regulated by CCR7 and its ligands CCL19/21 secreted by FRCs. As DCs are enriched in this area of the lymph node, T cells are able to probe DCs in search for their cognate antigens while migrating throughout the lymph node. (Bajénoff et al., 2006a; Link et al., 2007; Worbs et al., 2007) Autotaxin, expressed by FRCs, also regulates the motility of T cells by signaling through LPA receptors expressed on T cells (Katakai et al., 2014; Takeda et al., 2016). Unlike in HEVs, chemokines in the lymph node parenchyma do not activate lymphocyte integrins due to lack

of shear stress. Therefore, intranodal T cell migration is independent of integrins. (Woolf et al., 2007) Upon the entry of naïve B cells from the blood vasculature, they concentrate around HEVs, where they can interact with DCs leading to early activation of B cells (Qi et al., 2006). In contrast to T cells, naïve B cells are retained around HEVs for hours and they do not enter B cell follicles readily. B cells are known to migrate along the FRC network towards the lymph node cortex (Bajénoff et al., 2006a) and their subsequent entry into the follicles is partly regulated by G protein coupled receptor (GPCR) -signaling. Dissimilar to naïve B cells, activated B cells exit the perivascular area efficiently and migrate directly into the follicles. (Park et al., 2012). B cell migration is mainly regulated by the chemokine receptor CXCR5 expressed by B cells, and its ligand CXCL13 expressed by FDCs. CCR7 and CXCR4 have no role in regulating B cell migration in the lymph node parenchyma. Instead, signaling via the LFA-1/ICAM-1 pathway supports CXCR5 regulated migration, albeit integrin signaling is not required for stromal cell supported migration. (Ansel et al., 2000; Coelho et al., 2013)

2.3.4 Lymphocyte egress

The main molecules regulating lymphocyte egress from peripheral lymph nodes are S1P and its receptor $S1P_1$. S1P is produced intracellularly by all cells as an intermediate of sphingolipid metabolism, yet only extracellular S1P signals through its receptors. There are five different S1P receptors; all are GPCRs that signal via different Gαi subunits. Signaling can be efficiently abrogated with pertussis toxin. Of the five receptors, $S1P_1$ regulates cell egress. Its expression correlates negatively to the concentration of S1P, as even low S1P concentrations cause internalization of the receptor. (Cyster and Schwab, 2012) S1P is produced by sphingosine kinases 1 and 2, and its concentration is high in plasma and lymph. Although lymph is considered a filtration of plasma, plasma S1P is produced by red blood cells whereas LECs are responsible for producing S1P in lymph. (Pappu et al., 2007; Pham et al., 2010) In LECs, the extracellular secretion of S1P is regulated by the sphingolipid transporter Spns2 (Mendoza et al., 2012). Tissue S1P concentration is very low due to S1P lyase that is expressed by hematopoietic cells and rapidly degrades S1P in the lymph node (Schwab et al., 2005). Differences in S1P concentrations result in high expression of $S1P_1$ in lymphocytes in the lymph node, whereas lymphocytes in the blood stream and lymph downregulate the receptor due to ligand sensitization.

Early studies discovered lymphocyte egress to be abrogated when treating mice with FTY720, an S1P analogue functioning as an $S1P_1$ agonist. FTY720-treatment causes lymphatic sinuses to be cleared from lymphocytes in addition to

causing lymphopenia. In the lymph node, lymphocytes are retained at the abluminal side of the sinus lymphatics and are tightly jammed against the endothelium. The same is true for lymphocytes devoid of $S1P_1$. (Brinkmann et al., 2002; Mandala et al., 2002; Matloubian et al., 2004) FTY720, or fingolimod (sold as Gilenya), has proven to be a very effective immunosuppressive drug and it was the first oral drug available for the treatment of multiple sclerosis (Cyster and Schwab, 2012).

Successful lymphocyte egress is a combination of chemotactic egress promoting signals that overcome the opposing retention signals. CCR7 promotes T cell retention in the lymph node and S1P–$S1P_1$ signaling must overcome this for T cells to migrate into the lymphatic sinuses. T cells devoid of CCR7 exit more rapidly than their controls, and $S1P_1^{-/-}$ T cells are able to egress even when chemokine signaling is abrogated with pertussis toxin. (Pham et al., 2008) Similar to T cells, B cell entry into lymphatic sinuses is induced by the S1P–$S1P_1$ pathway. However, S1P chemotaxis plays no role in B cell egress. B cells that have occupied the lymph node for the most time gradually migrate from the central follicle to its rim, where they egress by entering the cortical sinuses. (Sinha et al., 2009)

Lymphocyte egress takes place at the cortical sinuses, which are located near HEVs and B cell follicles in the cortex of the lymph node. These sinuses are densely packed with lymphocytes and connect directly to the medullary sinus. Lymphocytes can cross into the cortical sinus at multiple locations and sometimes several cells enter the sinus from the same site. This led to the concept of gateways in the LEC layer, which may support lymphocyte migration more readily. Lymphocytes encountering the sinus often migrate along its abluminal side, probe the sinus lumen and thereafter decide whether to cross the LEC layer or not. (Grigorova et al., 2009, 2010; Sinha et al., 2009) Adhesion of lymphocytes to the cortical sinus LECs has been reported to be independent of $S1P_1$ signaling (Zhi et al., 2011). In fact, LFA-1 expressed by T cells mediates their adhesion and plays a role in deciding whether cells cross into the sinus, or whether they execute reverse migration back into the parenchyma (Reichardt et al., 2013). After crossing into the sinus, lymphocytes migrate along the sinus wall. There, they become captured by the lymphatic flow, which carries them to the medullary sinus. From thereon, lymphocytes get access to the subcapsular region surrounding the hilus area of the lymph node, and they drift with lymph into the efferent vessel leading to the next lymph node in the chain. (Grigorova et al., 2009)

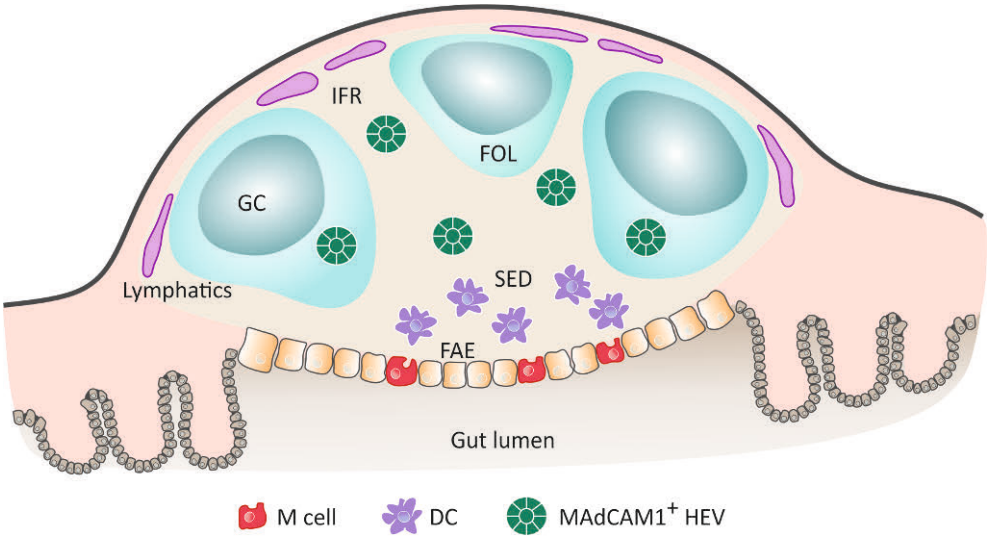

Figure 5. A schematic view of the murine Peyer's patch depicting its major structures. Peyer's patches constantly receive antigens from the intestinal lumen, which leads to prominent germinal centers (GC) in their B cell follicles (FOL). Peyer's patches lack afferent lymphatics, and antigens arrive through the follicle-associated epithelium (FAE) through specialized M cells. Dendritic cells (DC) populate the subepithelial dome (SED) close to the FAE, and they present antigens to lymphocytes in interfollicular regions (IFR) or at the borders of follicles. Lymphocytes enter the Peyer's patches via MAdCAM1$^+$ HEVs and egress from the efferent lymphatics.

2.4 Intestinal lymphocyte and antigen traffic

The intestine is constantly exposed to antigenic stimuli originating from diet and the commensal microbiota. It can also be invaded by pathogenic factors rather easily. Therefore, it is no surprise that the intestine is the largest compartment of the immune system harboring the largest amount of immune cells in the body. Immune cells are present mostly in the small intestine and colon, in which they populate the mucosal layer of the intestinal wall and contribute to maintaining homeostasis. The intestinal epithelium is populated by CD8$^+$ intraepithelial lymphocytes, whereas different subsets of CD4$^+$ T cells and plasma cells reside in the lamina propria. (Agace and McCoy, 2017) Gut associated lymphoid tissue (GALT) contains small, diffuse structures as well as its most defined structures, Peyer's patches, which are situated along the serosal layer of the intestine (Figure 5). Peyer's patches are comprised of large B cell follicles with reactive germinal centers (GC) and small T cell-rich IFRs. A unique structure of the Peyer's patches is the follicle-associated epithelium (FAE). It separates the Peyer's patch from the intestinal lumen and contains specialized microfold (M) cells involved in antigen transport. The subepithelial dome (SED) that harbors DCs and other immune cells is situated under the FAE. DCs internalize antigens and present them to T and B

cells in the SED. Unlike peripheral lymph nodes, Peyer's patches are devoid of afferent lymphatics and only contain efferent lymphatics, which convey cell egress. (Mowat and Agace, 2014; Reboldi and Cyster, 2016)

There are several lymph nodes draining the distinct regions of the gut. The chain of mesenteric lymph nodes (MLN) is comprised of many lymph nodes, which drain definite parts of the small intestine and colon. Regional draining of the gut allows for a lymph node specific cell composition in addition to a specific function. Lymph nodes draining the proximal part of the small intestine are tolerogenic and prevent food allergies, whereas lymph nodes draining the colon take part in pro-inflammatory responses. (Van den Broeck et al., 2006; Esterházy et al., 2019; Houston et al., 2016)

2.4.1 Transporting intestinal antigens

Intestinal antigens are acquired from the gut lumen, and they are transported to gut draining lymph nodes in the mesentery or into Peyer's patches. Antigens can be transcytosed across the intestinal epithelium by epithelial cells. Thereafter, antigens are actively taken up by DCs and mononuclear phagocytes in the lamina propria. DCs and other phagocytes can also directly sample antigens from the gut lumen. Epithelial villi have a small number of M cells that may play a role in transporting luminal antigens. In contrast, M cells in the Peyer's patch play a major role in transporting antigens from the intestine into the lymphoid tissue. Antigens are actively internalized by M cells via endocytosis, transcytosed and released into large pockets residing on the abluminal side of the cells. Immune cells, such as DCs and macrophages, can migrate into the M cell pockets, internalize, process the antigens and present them to T cells in the SED and IFR, or B cells in follicles. (Reboldi and Cyster, 2016; Schulz and Pabst, 2013) Activated B cells in the SED can also directly sample antigens from the basolateral pockets of the M cells (Komban et al., 2019). Similar to lymph nodes, Peyer's patches also contain a reticular conduit structure that regulates fluid flow from the intestine to the lymphoid tissue. Small antigens are also transported throughout the Peyer's patch by the conduit network. (Chang et al., 2019) Once antigens are available to DCs, antigen presentation and the following activation of lymphocytes and their effectory functions have the same basics as in the periphery, although molecules and pathways may be specific to the gut.

2.4.2 Immune cell migration in gut lymphoid tissues

Lymphocyte migration into gut draining lymph nodes of the mesentery and Peyer's patches occurs mainly via the blood vasculature. The adhesion cascade has the

same steps as in peripheral lymph nodes; however, there are several gut specific molecules that regulate migration. Selectins are involved in lymphocyte rolling on HEVs by adhering to MAdCAM-1, which is specifically expressed in HEVs of the Peyer's patch and partly in the MLN. Chemokine mediated activation of integrins is induced by CCL21–CCR7, CXCL12–CXCR4 and CXCL13–CXCR5 signaling. The subsequent lymphocyte arrest is mediated by the $\alpha_4\beta_7$ integrin, the receptor for MAdCAM-1. In Peyer's patches, B cells are more abundant than T cells in comparison to peripheral lymph nodes. Therefore, signaling via the CXCL13–CXCR5 pathway as well as high expression of $\alpha_4\beta_7$ integrin favors B cell entry into Peyer's patches. Immune cell homing to the small intestine epithelium and lamina propria is regulated by CCL25–CCR9 and $\alpha_4\beta_7$–MAdCAM-1 pathways. The specific gut-homing molecules expressed on lymphocytes are upregulated by DCs and stromal cells. (Habtezion et al., 2016; Reboldi and Cyster, 2016)

The S1P–$S1P_1$ pathway mainly regulates lymphocyte egress in Peyer's patches (Pham et al., 2010). Chemokines are also involved in enabling egress from Peyer's patches. CXCR4 on B cells and its ligand CXCL12 expressed by stromal cells promotes B cell entry into the efferent lymphatic vessels in Peyer's patches. Opposing signals are supplied by CXCL13 expressed by FDCs trying to retain B cells in the follicles. (Schmidt et al., 2013) Interestingly, lymphocyte egress can also be regulated by the interplay of the nervous system and chemokine receptors. In the MLN, stimulation of the β_2-adrenergic receptors on lymphocytes enhances retention signals. These signals are conveyed by the direct binding of the β_2-adrenergic receptors to CCR7 and CXCR4. Unlike in the Peyer's patches, CCR7 promotes B cell retention, whereas CXCR4 promotes T cell retention. (Nakai et al., 2014) This demonstrates the complexity of regulating cell egress in different tissues, and reveals that the same molecules may have distinct, tissue-specific roles in regulating immune cell homeostasis.

2.5 Inflammation induces changes in the lymph node

During acute inflammation, the role of the adaptive immunity starts with the delivery of foreign antigens or pathogens to the draining lymph node and the activation of lymphocytes. Activated T cells undergo clonal expansion producing a large number of lymphocytes specific for the recognized antigen. These newly developed effector cells leave the lymph node and migrate to the site of inflammation (e.g. skin) to exert their effectory functions. Upon antigen recognition, B cells become activated, develop into plasma cells, and start producing specific antibodies against the foreign antigen. To accommodate all of

the aforementioned, the lymph node stroma goes under drastic changes as lymph nodes expand 5-10 fold during inflammation. (Acton and Reis e Sousa, 2016)

During lymph node expansion BEC, LEC and FRC stromal cell compartments all undergo proliferation. HEVs dedifferentiate into an immature state and lose expression of HEV specific molecules. Expansion of the vascular compartment, which is necessary to support the increased entry of naïve lymphocytes to the lymph node, is regulated by DCs. DC-mediated signaling via the LTβR increases production of VEGF by FRCs, which promotes BEC proliferation. Permeability of blood vessels is increased during inflammation, which is also regulated by DCs. (Acton and Reis e Sousa, 2016; Dasoveanu et al., 2016; Thierry et al., 2019) The molecular composition of the endothelium changes as new players join in regulating the adhesion cascade and attracting other immune cells into the lymph node. (Ley et al., 2007; Muller, 2003; Salmi and Jalkanen, 2014) Simultaneously, lymphocyte egress is terminated causing total shutdown and retainment of lymphocytes in the lymph node to increase the likelihood of antigen recognition. During inflammation, T cells express CD69, which physically interacts with $S1P_1$, causes its internalization resulting in diminished T cell egress. After 3 days, T cells regain $S1P_1$ expression and are able to exit the lymph node and migrate to sites of inflammation. (Cyster and Schwab, 2012)

Similar to BECs, LECs also undergo proliferation during inflammation. Proliferation requires an effective T cell response that provides the necessary stimuli to the lymph node stroma to induce LEC expansion. Proliferating LECs can retain antigens archiving them for up to several weeks in the draining lymph node. Antigen archiving results in increased inflammatory responses during secondary challenge leading to increased immune protection. (Tamburini et al., 2014) Lymph node LECs may also harbor *Mycobacterium tuberculosis* bacteria supporting extrapulmonary tuberculosis (Lerner et al., 2016).

The reticular stroma reacts to facilitate the growing number of lymphocytes in the lymph node. During inflammation, DCs induce relaxation of FRCs to stretch the conduit and allow for expansion of the lymph node. FRC proliferation is further required for the stroma to increase in size and support the growing lymphocyte population. (Acton and Reis e Sousa, 2016; Dasoveanu et al., 2016) When inflammation is resolved, the lymph node stroma returnes to a resting state. Yet, the number of stromal cells remains elevated for several weeks after inflammation and antigens may reside in the lymph node for months. If inflammation is not resolved it becomes chronic, which involves the formation of tertiary lymphoid structures at extralymphoid sites in the periphery. Resolved inflammation leaves a scar damaging the local immune system, even if inflammation is resolved quickly. This scarring may have an effect on future immunological challenges and possibly even contribute to the development of chronic inflammation. (Acton and Reis e Sousa, 2016)

3 Aims

Lymphocyte migration and the delivery of foreign antigens into the lymph node is central for mounting adaptive immune responses. Migrating immune cells and antigens both encounter the lymphatic endothelium when entering peripheral lymph nodes. Yet, the role of lymphatic endothelial cells in regulating cell migration or antigen transport is not fully comprehended. These studies were performed to elucidate the different regulatory functions of the lymph node lymphatics. The precise aims were:

1. Investigating molecular differences in lymph node afferent and efferent LECs and the role of lymphatic Msr1 in regulating lymphocyte migration via the afferent lymphatics
2. Studying the role of the efferent lymphatic marker Robo4 in regulating lymphocyte migration via the blood vasculature and lymphocyte egress in Peyer's patches
3. Describing the mechanism of the delivery of intact lymph-borne antibodies and other large antigens into the draining lymph node parenchyma

4 Materials and Methods

4.1 Animals (I–III)

Animal models were used in all publications of this thesis. Mice were housed in the animal facilities of the University of Turku, Finland and RWTH Aachen University, Germany. Animals were housed in open or individually ventilated cages with a light-dark cycle of 12 hours. All test subjects had *ad libitum* access to dried chow pellets and water. All animal experimentation was approved by the Ethical Committee for Animal Experimentation in Finland and Germany. Experiments were conducted according to the rules and regulations of The Finnish Act on Animal Experimentation (497/2013) with respect to the 3R-principle and performed under the following animal licences: 3791/04.10.03/2011, 5587/04.10.07/2014, 6211/04.10.07/2017, 5762/04.10.07/2017 and 84-02.04.2014.A330.

Wild type (WT) strains of several different backgrounds were used for *in vivo* experiments, or as controls for knockout (KO) strains. C57BL/6J, C57BL/6N and BALB/C mice were purchased from Jackson Laboratories, Janvier Labs and Charles River Laboratories. Congenic CD45.1^{+} B6.SJL-*Ptprc*^{a} *Pepc*^{b}/BoyJ were purchased from Jackson Laboratories. Genetically modified mouse models include B6.Cg-*Msr1*^{tm1Csk}/J (Msr1 KO, stock 006096), *Cav*$^{1tm1Mls/J}$ (Caveolin 1 (Cav1) KO, stock 004585), *FcRn*$^{-/-}$ (Roopenian et al., 2003) and OTII mice (which express transgenic αβ T cell receptors specific for chicken ovalbumin (OVA)) that were purchased from Jackson Laboratories. 129S6(B6)-*Robo4*^{tm1Lex}/Mmucd (Robo4 KO, stock 032543-UCD) and their WT controls with the same genetic background were purchased from Lexicon genetics Inc. and bred as separate lines.

4.2 *In vivo* administered reagents (II, III)

Fluorescent antibodies and dextrans were filtered using 10-kDa cutoff centrifugal units (MRCPRT010, Millipore) to remove any free fluorochromes and to concentrate antibodies. Mixtures of antibodies, 70 kDa TRITC dextran (D1818, Invitrogen) or 500 kDa FITC dextran (D7136, Invitrogen; II, III; see Table 2) were injected s.c. into the hind paws of recipient mice in volumes of 1–20 µl using

Microfine Demi 0.3 ml syringes (BD). For blocking experiments, the Fc binding function-blocking antibody was administered alone or with mouse immunoglobulins (010-0102-0005 Rockland) before, and with the test antibody mixture to block nonspecific binding to Fc receptors. In competition experiments, an unlabeled ratIgG2a monoclonal antibody (9B5 targeting human CD44) or mouse immunoglobulins were administered with test antibodies s.c. to recipient mice. During injections, the mice were under light isoflurane anesthesia. At predetermined timepoints, the mice were sacrificed; draining and non-draining lymph nodes were harvested and processed for microscopy or flow cytometry.

To analyze vascular distribution of antibodies or to label BECs (II, III), fluorescent antibodies were administered to recipient mice with intravenous (i.v.) injections by injecting antibodies into the tail vein. After selected timepoints, mice were sacrificed and lymph nodes or Peyer's patches were processed for imaging or flow cytometry. For detecting opposing structures of blood vessels, CD31-A594 was administered s.c. followed by i.v. injections of PLVAP-A488.

Fluorescein isothiocyanate (FITC, Sigma) was injected into Peyer's patches to analyze turnover of lymphocytes (II). Mice were subjected to general anesthesia and the small intestine was surgically exposed. FITC was injected into 4-5 exposed Peyer's patches/mouse (1 mg/ml) using fine glass capillaries connected to a microinjector. After the procedure, the small intestine was returned to the abdomen and surgical wounds were closed. Peyer's patches were analyzed with flow cytometry after 24 hours.

Table 2. *In vivo* administered antibodies against mouse antigens.

ANTIBODY	ROUTE	CLONE	CAT. NO.	COMPANY	PUB.
B220-eFluor 570	s.c.	RA3-6B2	41-0452-80	eBioscience	III
B220-Pacific Blue	s.c., i.v.	RA3-6B2	558108	BD	III
CD3ε-FITC	s.c.	145-2C11	553062	BD	III
CD4-AF647	s.c.	RM4-5	557681	BD	III
CD4-FITC	s.c., i.v.	RM4-5	11-0042-81	eBioscience	III
CD4-PE	s.c.	RM4-5	553048	BD	III
CD8α-AF488	s.c.	53-6.7	557668	BD	III
CD8α-FITC	s.c.	53-6.7	553031	BD	III
CD8α-FITC	s.c.	3.155	FCMAB427F	Milli-Mark	III
CD8α-HRP	s.c.	53-6.7	MA1-10301	Thermo Scientific	III
CD11c-AF647	s.c., i.v.	N418	117314	BioLegend	III
CD16/CD32	s.c.	2.4G2	553142	BD	III
CD16/CD32	s.c.	2.4G2	BE0307	Bio X Cell	II
CD19-FITC	s.c.	MB19-1	11-0191-81	Invitrogen	III
CD28	s.c.	37.51	16-0281	eBioscience	III

ANTIBODY	ROUTE	CLONE	CAT. NO.	COMPANY	PUB.
CD28	s.c.	B665	LS-C58166	LSBio	III
CD31-AF488	s.c.	MEC13.3	102514	BioLegend	III
CD31-AF594	s.c., i.v.	MEC13.3	102520	BioLegend	III
CD31-Biotin	s.c.	MEC13.3	553371	BD	III
CD31-DyL550	s.c.	MEC7.46	NB100-1642R	Novus Biologicals	III
CD45.2-BV711	s.c.	104	563685	BD	III
CD62L	s.c.	MEL-14	Gallatin, Weissman and Eugene, 1983	In house	III
CD62L $(Fab)_2$	s.c.	MEL-14	Gallatin, Weissman and Eugene, 1983	In house	III
CD90.2	s.c.	30-H12	105315	BioLegend	III
Collagen I	s.c.	pAb	AB765P	Millipore	III
ER-TR7-DyL405	s.c.	ER-TR7	NB100-64932V	Novus Biologicals	III
F4/80-AF647	s.c.	CI:A3-1	MCA497A647	Biorad	III
H-$2K^b$-FITC	s.c.	AF6-88.5	116505	BioLegend	III
Ly6C-APC	s.c.	HK1.4	128016	BioLegend	III
MAdCAM-1	i.v.	Meca367	Berg *et al.*, 1993	In house	II
PNAd	s.c.	Meca79	Streeter, Rouse and Butcher, 1988	In house	III
PLVAP-AF488	s.c., i.v.	Meca32	120506	BioLegend	III
Robo4-AF647	s.c.	FuRFM7	Fair-Mäkelä *et al.*, 2020	In house	II
Stabilin-1	s.c.	9-11	Palani *et al.*, 2011	In house	III
Stabilin-1 $(Fab)_2$	s.c.	9-11	Palani *et al.*, 2011	In house	III
TCR β chain-PerCP Cy5.5	s.c.	H57-597	560657	BD	III
Chicken IgG-AF647	s.c.	pAb	A21463	Invitrogen	III
Donkey IgM-AF647	s.c.	pAb	715-605-140	Jackson	III
Goat IgM-AF647 (Fab)	s.c.	pAb	115-607-020	Jackson	III
Hamster IgG1-FITC	s.c.	A19-3	553971	BD	III
Hamster IgG2a-PerCP Cy5.5	s.c.	B81-3	560562	BD	III
Rabbit IgM-AF647	s.c.	pAb	315-605-020	Jackson	III
Rat IgG2a	s.c.	R35-95	553926	BD	III
Rat IgG2a	s.c.	Hermes-1	Jalkanen *et al.*, 1987	In house	III
Rat IgG2a-AF488	s.c.	RTK2758	400525	BioLegend	III
Sheep IgG-AF488	s.c.	pAb	515-545-062	Jackson	III

4.3 *In vivo* experiments (I–III)

4.3.1 Adoptive transfer assays (I, II)

Lymphocytes were mechanically isolated from the spleen, skin-draining and cervical lymph nodes of WT donor mice, and labeled with 0.5 μM CellTrace CFSE (C34554, Invitrogen). $10x10^6$ or $1x10^6$ cells were injected s.c. into the footpads of recipient mice; Msr1 KO, Robo4 KO and their WT controls, while mice were under light isoflurane anesthesia. Lymphocytes were let to migrate into the draining popliteal lymph nodes (and further to the sacral and iliac lymph nodes) for 4.5 or 12 hours. The number of transferred cells was analyzed with flow cytometry, and their localization was examined with fluorescence imaging.

For studying the role of vascular Robo4 in lymphocyte migration, lymphocytes were isolated from the skin-draining, mesenteric and cervical lymph nodes of congenic $CD45.1^+$ donor mice. Lymphocytes ($12x10^6$ cells/recipient) were injected i.v. into $CD45.2^+$ Robo4 KO and their WT controls. After 2 or 18 hours, mice were sacrificed and the number and localization of adoptively transferred cells was analyzed from peripheral lymph nodes (inguinal, axillary, brachial), MLN, Peyer's patches and peripheral blood using flow cytometry and fluorescence imaging.

4.3.2 Activation of T cells (III)

Activation of T cells was performed by administrating anti-TCR and anti-CD3 antibodies, or their isotype specific controls, by s.c. injections into the footpads of recipient mice. After 5 min or 18 hours, the draining and non-draining popliteal lymph nodes were isolated and T cell activation was determined by examining the early activation markers CD69 and CD25 with flow cytometry.

In another set of experiments, splenocytes were isolated from $CD45.2^+$ OTII donor mice (specifically recognizing OVA), enriched for naïve $CD4^+$ T cells with the EasySep Mouse Naïve T cell isolation kit (19765, Stemcell Technologies) and labeled with 1 μm CellTrace CFSE. $5x10^6$ lymphocytes were adoptively transferred into $CD45.1^+$ recipient mice by i.v. injections, and they were allowed to distribute to secondary lymphoid organs for 18 hours. To stimulate T cell proliferation, an agonistic anti-CD28 antibody (clone D665) or an inhibitory antagonistic anti-CD28 antibody (clone 37.51) was administered s.c. to the recipient mice along 2 μg of OVA (321000, Hyglos). OVA combined with the adjuvant, lipopolysaccharide (LPS, L3024, Sigma) was used as a positive control. After 3 days, the draining and non-draining popliteal lymph nodes were isolated and the proliferation of the transferred OTII cells was analyzed with flow

cytometry. Cells were separated into $CFSE^{hi}$ (undivided) or $CFSE^{lo}$ (divided ≥5 times), and the absolute number of any $CFSE^{+}$ cells was determined from the whole tissue.

4.3.3 Uptake of antigens (III)

To analyze the uptake of foreign antigens after s.c. injections, 2 µg of recombinant human AOC3 protein (150-16, PeproTech) labeled with Alexa Fluor 647 was injected s.c. into recipient mice. Draining popliteal lymph nodes as well as lymph nodes from untouched mice were collected after 2 hours and processed for flow cytometry or microscopic analyses.

4.3.4 Depletion of macrophages (I, III)

Resident lymph node macrophages were depleted with s.c. injections of clodronate or vehicle containing liposomes to the footpads of recipient mice (Rooijen and Sanders, 1994). When antibodies were also administered to the same footpad, clodronate was administered to the anterolateral surface of the tibia and antibodies were injected into the footpad of the same paw three days later.

4.3.5 Blocking of endocytosis (III)

Endocytosis was blocked using the following chemical inhibitors targeting clathrin (Pitstop 2 (ab120687, Abcam) and monodansylcadaverine (MDC, D4008, Sigma)), dynamin (Dyngo-4a (A511904, Sigma) and Dynole 34-2 (ab120463, Abcam)), macropinocytosis (EIPA [5-(*N*-ethyl-*N*-isopropyl amiloride] (A3085, Sigma)) and a chemical stabilizing interendothelial junctions (adrenomedullin (NC0928729, Phoenix Pharmaceuticals)). Popliteal lymph nodes were surgically exposed while mice were under general anesthesia. The inhibitors and their appropriate negative controls were administered directly onto the exposed popliteal lymph nodes as fresh droplets for 15 min. Test antibodies were injected s.c. into the footpads of treated mice and lymph nodes were collected after 5 min.

4.3.6 Monoclonal antibody production (II)

A Robo4 KO mouse was immunized with 5 µg recombinant murine Robo4 (MBS2546674, MyBioSource) in Freund's Incomplete Adjuvant by s.c. injections into the footpad once a week for three weeks. The draining popliteal lymph node was collected, fused to the mouse myeloma cell line SP2/0 followed by the production of hybridoma cell lines using the ClonaCell-HY Hybridoma Kit (03800,

Stemcell Technologies) according to the manufacturer's instructions. Cell lines were cultured in Dulbecco's modified eagle medium (D6429, Sigma), positive clones detecting Robo4 were adapted to serum free CD Hybridoma medium (11279-023, Gibco) and the buffer was changed to PBS while concentrating the antibodies with 100-kDa cutoff centrifugal filter units (UCF910024, Millipore). Isotypes were determined using the Pierce Rapid Antibody Isotyping Kit plus Kappa and Lambda – Mouse (26179, Thermo Scientific). The antibody was validated with an enzyme-linked immunosorbent assay (ELISA), flow cytometry and immunofluorescence imaging using Robo4 overexpressing Hek cells.

4.4 Flow cytometry (I–III)

4.4.1 Preparing single-cell suspensions (I–III)

Single-cell suspensions were prepared from peripheral lymph nodes (PLN, I–III), MLN (II), Peyer's patches (II) and the bone marrow (II). Lymphocytes were extracted from lymph nodes and Peyer's patches with mechanical homogenization using cell strainers, followed by washing and filtering the samples to remove tissue remnants. To isolate B cell progenitors from the bone marrow (II), femurs were collected and crushed with a mortar in Hanks' buffered salt solution (HBSS) supplemented with 2% fetal calf serum (FCS). When analyzing peripheral blood, erythrocytes were lysed with hypotonic solutions (II).

For detection of antigen presenting cells (III), lymph nodes were mechanically minced into small pieces that were enzymatically digested in HBSS containing collagenase D (1 mg/ml) and DNase I (50 µg/ml) for 30 min at +37 °C. Endothelial cells were isolated from peripheral lymph nodes, MLN, Peyer's patches and the skin (I, II) with enzymatic digestion. The samples were mechanically minced and incubated several times with an enzymatic cocktail containing Liberase (25 µg/ml) and DNase I (100 µg/ml) or collagenase P (0.2 mg/ml) and neutral protease (0.8 mg/ml) in phosphate buffered saline (PBS) at +37 °C with gentle agitation.

4.4.2 Staining and acquiring samples (I–III)

After obtaining single-cell suspensions, samples were stained with primary antibodies followed by fluorescently labeled secondary antibodies, or with fluorescently conjugated antibodies (Table 3). Unspecific antibody staining was blocked with an anti-CD16/CD32 Mouse BD Fc Block (BD 553141) or 5% rat serum. Antibodies were added to cells, incubated on ice and samples were fixed using paraformaldehyde (PFA) supplemented buffer. The apoptotic rate of lymphocytes was analyzed using the Annexin V staining according to the

manufacturer's instructions. For analyzing samples with cytometers (I-III), cells were acquired using a LSR II (I) or LSR Fortessa (II, III) both from BD. Samples were analysed using FlowJo software (FlowJo, BD).

4.4.3 Fluorescence-activated cell sorting (I)

Stromal cells were isolated from enzymatically digested peripheral lymph nodes and MLNs collected from C57BL/6J mice. Samples were stained for the appropriate markers (Lineage cocktail, podoplanin, CD73 or their isotype controls) and 7-AAD to exclude dead cells. For isolating mouse LECs for cell culturing, samples were stained with Lineage cocktail, CD45, CD11b, podoplanin, CD31 and LYVE1. Cells were sorted with FACSAria II (BD), $CD73^+$ or $CD73^-$ LECs ($Lineage^-$ $podoplanin^+$) were collected for RNA extraction and $Lineage^-$ $CD45^-$ $CD11b^-$ $podoplanin^+$ $CD31^+$ $LYVE1^+$ cells were subjected for cell culturing.

Table 3. Antibodies against mouse antigens used in flow cytometry and cell sorting.

ANTIBODY	CLONE	CAT. NO.	COMPANY	PUB.
Annexin V-PE	-	556422	BD	II
B220-Pacific Blue	RA3-6B2	558108	BD	I, III
B220-PE	RA3-6B2	561878	BD	I
B220-BV421	RA3-6B2	562922	BD	II
CD1d-PerCP Cy5.5	1B1	562713	BD	II
CD3-AF647	17A2	557869	BD	II, III
CD3-PerCP Cy5.5	17A2	100218	BioLegend	III
CD4-AF647	RM4-5	557681	BD	I, III
CD4-APC Cy7	GK1.5	552051	BD	I, II, III
CD4-BV510	RM4-5	100553	BioLegend	II
CD4-FITC	RM4-5	11-0042-81	BioLegend	III
CD4-V500	RM4-5	560782	BD	III
CD5-PE	53-7.3	553023	BD	II
CD8α-BV650	53-6.7	563234	BD	I, II, III
CD8α-BV711	53-6.7	100747	BD	II
CD8α-FITC	53-6.7	553031	BD	III
CD8α-FITC	3.155	FCMAB427F	Milli-Mark	III
CD8α-PerCP Cy5.5	53-6.7	551162	BD	I, III
CD11b-APC	M1/70	553312	BD	I
CD11b-APC Cy7	M1/70	561039	BD	I, II
CD11b-PE	M1/70	553311	BD	III
CD11c-BV421	HL3	562782	BD	III
CD16/CD32	2.4G2	553141	BD	I, II, III

ANTIBODY	CLONE	CAT. NO.	COMPANY	PUB.
CD19-APC Cy7	1D3	557655	BD	II
CD19-BV421	6D5	115537	BioLegend	II
CD19-BV510	1D3	562956	BD	II
CD24-BV650	M1/69	563545	BD	II
CD25-PE	PC61	553866	BD	III
CD31-APC	MEC13.3	102510	BioLegend	I, II
CD40-PE CF594	3/23	562847	BD	II
CD43-BV605	S7	563205	BD	II
CD44-APC	IM7	17-0441-82	eBioscience	II
CD44-PerCP Cy5.5	IM7	560570	BD	II
CD45-APC Cy7	30-F11	557659	BD	I, II
CD45-FITC	30-F11	553079	BD	II
CD45-PE	30-F11	553081	BD	II
CD45-V500	30-F11	561487	BD	III
CD45.1-FITC	A20	553775	BD	II
CD45.2-APC	104	558702	BD	II, III
CD45.2-PerCP Cy5.5	104	552950	BD	III
CD62L-BV421	MEL-14	104436	BioLegend	II
CD62L-AF647	MEL-14	104417	BioLegend	II
CD69-APC	H1.2F3	17-0691-80	eBioscience	I
CD73-PE	TY23	550741	BD	I
CD95-PE	Jo2	554258	BD	II
CD169-BV605	3D6.112	142413	BioLegend	III
Endomucin	V.5C7	MAB2624	Millipore	I
Endomucin-eFluor 660	V.7C7	50-5851	eBioscience	I
F4/80-AF488	BM8	MF48020	Invitrogen	III
GL7-AF647	GL7	561529	BD	II
Lineage Ab cocktail-APC	-	558074	BD	I
Lineage Ab cocktail isotype-APC	-	558074	BD	I
LYVE1	pAB	103-PA50	ReliaTech GmbH	I
LYVE1-PE	223322	FAB2125P	R&D Systems	I, II
MHCII-PE Cy7	M5/114.15.2	25-5321-82	eBioscience	II, III
Podoplanin-PE Cy7	8.1.1	127412	BioLegend	I, II
Robo4	274940	MAB5004	R&D Systems	II
Robo4	FuRFM7	Fair-Mäkelä *et al.*, 2020	In-house	II
Robo4-PE	274914	FAB50041P	R&D Systems	II
Streptavidin-AF488	-	S11223	Invitrogen	I
Mouse IgD-BV786	11-26c.2a	563618	BD	II
Mouse IgG1	Ak1	-	InVivo BioTech	II

ANTIBODY	CLONE	CAT. NO.	COMPANY	PUB.
Mouse IgM APC-eFluor780	II/41	47-5790-80	eBioscience	II
Rat IgG1-FITC	RTK2071	400405	BioLegend	I
Rat IgG2a-eFluor 660	eBR2a	50-4321-82	eBioscience	I
Rat IgG2a-FITC	R35-95	553929	BD	I, II
Rat IgG2a-PE	R35-95	553930	BD	I, II
Rat IgG2a-PE	54447	IC006P	R&D Systems	II
Rat IgG2b-FITC	A95-1	556923	BD	I
Donkey anti-mouse IgG-AF647	pAb	A31571	Invitrogen	II
Goat anti-rat IgG-AF488	pAb	A11006	Invitrogen	III
Goat anti-rat IgG-FITC	pAb	F6258	Sigma-Aldrich	I, II

4.5 Processing samples for microscopy (I–III)

Lymph nodes, MLN and Peyer's patches were harvested from mice and embedded in optimal cutting temperature (OCT) medium. Peyer's patches were collected attached to the small intestine and fixed with 4% PFA for 2–4 hours at +4 °C, washed with PBS twice for 10 min and incubated in 30% sucrose overnight. Popliteal LNs and Peyer's patches were embedded in OCT in a predetermined orientation to produce transverse sections of the tissues. For some stainings, Peyer's patches were mounted without PFA fixing. Tissue blocks were snap frozen using dry ice, stored at –70 °C and used for cutting 6 µm sections. In some cases, lymph node samples were embedded in paraffin. Tissue sections were stained with primary antibodies followed by fluorescently conjugated secondary antibodies, or fluorescently conjugated antibodies using predetermined antibody concentrations at room temperature (Table 4). Sections were overlaid with ProLong Gold Antifade Mountant with or without DAPI (Invitrogen, P36931 and P10144, respectively). Late apoptotic cells were stained from fixed Peyer's patch sections using the Click-IT Plus TUNEL Assay (C10618, Invitrogen) according to the manufacturer's instructions.

Lymph nodes containing s.c. delivered antibodies were not subjected to any additional antibody staining unless indicated (*ex vivo* staining). When detecting the unlabeled, free antibody (rat IgG2a), the antibody was fixed *in situ*. Thirty seconds after the injection of the free antibody, 4% PFA was injected s.c. into the same footpad. Ninety seconds later, the mice were sacrificed and lymph nodes were processed for microscopy. Before staining with antibodies, the sections were further fixed with 4% PFA for 5 min at +4 °C, washed with PBS and quenched with 0.1 M glycine. Lymph nodes s.c. injected with lysine-fixable dextrans were similarly fixed *in situ* and *ex vivo*. Popliteal lymph nodes from untouched mice were fixed identically to serve as proper controls.

Staining for the early endosomal marker EEA1 was perfomed the following way: frozen sections were washed, fixed with 4% PFA for 15 min at room temperature, washed twice with PBS and quenched with 50 mM NH_4Cl for 15 min at room temperature. Samples were blocked with 30% horse serum and permeabilized with 0.3% Triton-X. Sections were stained with the EEA1 antibody followed by Alexa Fluor 546-conjugated anti-rabbit IgG antibody. HRP-conjugated antibodies were administered s.c. and visualized using DAB as a substrate.

For electron microscopy, samples were fixed, dehydrated and embedded with 45359 Fluka Epoxy Embedding Medium kit. For immunoelectron microscopy, the free antibody was injected s.c. into recipient mice. After 2 min, the mice were perfused with warmed PBS and tissues were perfusion fixed. Draining lymph nodes were collected, fixed and incubated in 2.1 M sucrose at +4 °C overnight for cryoprotection. The lymph nodes were snap-frozen, cut into 10 µm sections and stained with 10 nm gold-conjugated anti-rat IgG (EM.GAT10, BBI Solutions) Samples were further processed for imaging and embedded on microscope slides. Using an ultramicrotome, 70 nm sections were cut and stained with 1% uranyl acetate and 0.3% lead citrate for both transmission and immunoelectron microscopy.

4.6 Imaging and image analysis (I–III)

4.6.1 Acquiring images (I–III)

Fluorescence images were acquired using an Olympus BX60 fluorescence microscope (Tokyo, Japan) equipped with an UPlanFI 20x/0.50 or UPlanFI 40x/0.75 objective. Alternatively, images were acquired with a Nikon Eclipse TI-E fluorescence microscope with a Plan Apo lamda 20x/0.80 objective. Electron microscopy samples were examined using a JEM-1400 Plus transmission electron microscope (JEOL).

Confocal images were imaged using a LSM780 or LSM880 confocal microscope (Carl Zeiss) with Plan-Apochromat 20x/0.8 and c-Apochromat Korr M27 40x/1.20 objectives. Intracellular $EEA1^+$ vesicles were imaged with the LSM880 confocal microscope using an Airyscan detector (Carl Zeiss) and a C Plan-Apochromat 63x/1.4 oil objective. Images were acquired with the Zen 2010 of Zen 2.3 SP1 FP2 software (Carl Zeiss). The slice thickness was determined by adjusting the pinhole and the same thickness was used for each channel. In some cases, images were acquired with the Intelligent Imaging Innovations (3i) Marianas Spinning disk confocal microscope (Intelligent Imaging Innovations) with the Plan Apochromat 20x/0.8 N.A. objective and SlideBook 6 software.

4.6.2 Image analysis (I–III)

Image analyses were performed with ImageJ software (NIH). Linear brightness adjustments and background subtraction was applied equally to all images and their controls (I–III). The shortest distance of the adoptively transferred $CFSE^+$ lymphocytes from the SCS was measured by hand with Zen 2012 software (Carl Zeiss) while omitting lymphocytes in the medullary sinus from the analyses (I).

Mean fluorescence intensity was analyzed from defined zones starting from the SCS and proceeding towards the lymph node parenchyma after s.c. injections of free monoclonal antibody or dextrans (III). Localization of the free antibody in respect to conduit markers across reference lines was analyzed using the ImageJ plugin, RGB Profiler. In these analyses, the core of the conduit was defined as collagen I signal bordered by ER-TR7 signal (III). $EEA1^+$ vesicular structures and free antibody vesicles from the $CD31^+$ SCS floor LECs were analyzed using Imaris 8.1.2 software (Bitplane). Free antibody vesicles were detected with the 'Spots' tool and the surface of $EEA1^+$ vesicles was modeled using the 'Surface' tool. The free antibody vesicles in direct contact or immediate vicinity of $EEA1^+$ structures were manually enumerated (III).

For analyzing the areas of any selected positive signal, the appropriate signal determined by thresholding was analyzed in relation to the region of interest. Such image analyses were utilized when analyzing the anatomic localization of lymphocytes, blood and lymphatic vessels, adoptively transferred $CD45.1^+$ cells, $TUNEL^+$ apoptotic cells and collagen I^+ signal (II).

4.6.3 Tissue whole mount imaging (I, III)

For whole mount imaging of skin lymphatics (I), ears were collected from Msr1 KO and WT mice. Dorsal halves of the mechanically separated ears were fixed with 2% PFA, blocked for nonspecific staining with 1% normal mouse serum and 0.5% FCS in PBS-MT (PBS with 1% skim milk and 0.4% Triton X-100 (Sigma)) and stained for Msr1 and LYVE1. Ears were mounted onto microscopic slides and topped with coverslips. Samples were imaged using the Intelligent Imaging Innovations (3i) Marianas Spinning disk confocal microscope and the 20x/0.8 objective.

For whole mount imaging of the popliteal lymph nodes (III), 10 µg of fluorescently conjugated antibodies were injected s.c. into recipient mice and antibodies were let to drain to the lymph node for 30 min. Lymph nodes were harvested, fixed and dehydrated with methanol. For optical clearing, lymph nodes were incubated with 50% BABB diluted in MetOH (BABB was prepared by mixing one part benzyl alcohol with two parts benzyl benzoate) followed by 100% BABB until the tissue was clear. Samples were imaged using the LSM780 confocal

microscope (Carl Zeiss) and Plan-Apochromat 20x/0.8 objective by taking z stacks with a 5 µm slice thickness. Background subtraction and linear brightness adjustments were performed with the Imaris 8.1.2 software (Bitplane), which was also used to create a three-dimensional image reconstruction.

Table 4. Antibodies against mouse antigens used in microscopy, adhesion assays and ELISA.

ANTIBODY	CLONE	CAT. NO.	COMPANY	PUB.
B220-Pacific Blue	RA3-6B2	558108	BD	I, II
B220-eFluor 570	RA3-6B2	41-0452-80	eBioscience	II, III
CD4-AF647	RM4-5	557681	BD	I, II
CD8α-AF488	53.6-7	557668	BD	I, II
CD11c-FITC	N418	117306	BioLegend	III
CD19	6D5	115502	BioLegend	II
CD19-AF647	6D5	115522	BioLegend	II
CD31-AF488	MEC13.3	102514	BioLegend	I, II, III
CD31-AF594	MEC13.3	102520	BioLegend	III
CD31-AF647	390	102415	BioLegend	III
CD31-APC	MEC13.3	102510	BioLegend	III
CD44-FITC	IM7	553133	BD	II
CD45.1-Biotin	A20	110704	BioLegend	II
CD62L	MEL-14	(Gallatin et al., 1983)	In house	II
CD169-FITC	3D6.112	MCA884F	Biorad	I, III
CD204-AF488	2F8	MCA1322A488	Biorad	I
CD204-AF647	2F8	MCA1322A647	Biorad	I
Collagen I	pAb	AB765P	Millipore	II, III
EEA1	pAb	C45B10	Cell Signaling Technology	III
Endomucin	V.57C	MAB2624	Millipore	I
ER-TR7-DyL 405	ER-TR7	NB100-64932V	Novus Biologicals	III
F4/80-PE Texas Red	BM8	MF48017	Invitrogen	I
IgD-AF647	11-26c.2a	405708	BioLegend	II
LYVE1	pAb	103-PA50	ReliaTech GmbH	II
LYVE1	223322	FAB2125G	BD	I
LYVE1-AF488	ALY7	53-0443-82	eBioscience	II
Ly6C-APC	HK1.4	128016	BioLegend	III
MAdCAM-1	Meca367	Berg *et al.*, 1993	In house	II
Msr1	2F8	LS-C124019	LSBio	I
Msr1	pAb	GTX51749	GeneTex	I
PLVAP	Meca32	Hallmann *et al.*, 1995	In house	I, II

ANTIBODY	CLONE	CAT. NO.	COMPANY	PUB.
PNAd	Meca79	Streeter, Rouse and Butcher, 1988	In house	III
Podoplanin	8.1.1	ab111127	Abcam	I
Robo4	FuRFM7	Fair-Mäkelä *et al.*, 2020	In house	II
Robo4	pAb	bs-5795r	Bioss Antibodies	II
Siglec-1	3D6.112	MCA884EL	Biorad	I
Smooth muscle actin-Cy3	1A4	C6198	Sigma-Aldrich	III
Donkey anti-goat IgG AF488	pAb	A11055	Invitrogen	II
Donkey anti-goat IgG AF546	pAb	A11056	Invitrogen	I
Donkey anti-goat IgG AF633	pAb	A21082	Invitrogen	II
Goat anti-hamster IgG AF488	pAb	A21110	Invitrogen	I
Goat anti-hamster IgG AF546	pAb	A21111	Invitrogen	I
Goat anti-mouse IgG AF488	pAb	A11029	Invitrogen	II
Goat anti-rabbit IgG AF488	pAb	A11034	Invitrogen	I
Goat anti-rabbit IgG AF546	pAb	A11035	Invitrogen	I, II, III
Goat anti-rabbit IgG AF633	pAb	A21071	Invitrogen	III
Goat anti-rat IgG AF488	pAb	A11006	Invitrogen	I, II, III
Goat anti-rat IgG AF546	pAb	A11081	Invitrogen	I, II
Goat anti-rat IgG AF647	pAb	A21247	Invitrogen	I
Goat anti-rat IgM AF488	pAb	A21212	Invitrogen	III

4.7 Laser capture microdissection (I)

Peripheral lymph nodes collected from C57BL/6J mice were snap-frozen, stained with CD31 and F4/80 to determine endothelial cells and macrophages, and used for exctraction of LECs with laser capture microdissection. Cells were excised from the SCS and cortical sinus region representing the afferent and efferent lymphatics by using a Zeiss PALM MicroBeam instrument and a 40x objective. Dissected cells were collected into adhesive caps containing lysis buffer and subjected for RNA extraction.

4.8 Microarray analysis (I)

LECs representing the afferent and efferent lymphatics of the lymph node were isolated by flow cytometry and laser capture microdissection and used for performing a genome-wide microarray assay. RNA extraction, translation into complementary DNA and hybridization to the Agilent Whole Mouse Genome Oligo Microarrays 8x 60 K was performed at Miltenyi Biotec. Appropriate bioinformatic analyses were conducted to analyze differentially expressed genes

between the afferent and efferent lymphatics. The data obtained from the microarray is deposited to the GEO archive (GSE 68371).

4.9 *In vitro* experiments

4.9.1 Human material (I, II)

Lymph nodes and appendix samples were collected from the surplus tissues of patients undergoing surgical operations. Tissues that appeared to be normal after macroscopic and microscopic inspection were used for further analyses. Mononuclear cells were isolated from healthy volunteers with the appropriate permission granted by the Ethical Committee of the Turku University Hospital.

4.9.2 *Ex vivo* adhesion assays (I, II)

Human lymphocytes or CFSE labeled mouse lymphocytes were incubated on freshly cut frozen sections of human or mouse lymph nodes, or primary mouse LECs treated with blocking antibodies against Msr1, Robo4 or negative controls (see Tables 4 and 5). Lymphocytes were let to bind to lymph nodes for 5 min under rotation, 15 min at steady conditions, 5 min under rotation and 15 min at steady conditions at +7 °C. Mouse lymphocytes were incubated on lymph nodes for 30 min at +7 °C under rotation. Unbound cells were decanted off and bound cells were fixed with 1% glutaraldehyde. The number of bound lymphocytes was enumerated manually under dark-field microscopy and control values were set to 100% by definition.

The extracellular domain of murine Msr1 cloned from murine Msr1 cDNA (OMG222674, Origene) was fused to the human IgG2 Fc tail to generate an Msr1-Fc chimera. The Msr1-Fc chimera or control Ig were incubated with lymphocytes isolated from mouse lymph nodes for 30 min at RT. Binding of the chimera was detected with a biotinylated anti-human Ig antibody followed by Alexa Fluor 488 conjugated Streptavidin (see Table 5). Lymphocytes were stained with lymphocyte markers and samples were ran with LSR Fortessa (BD).

4.9.3 Culturing primary cells (I)

Mononuclear cells were isolated from the peripheral blood of healthy donors and enriched for monocytes using negative or positive selection kits (Miltenyi Biotec). Monocytes were polarized towards M1 or M2 macrophages by culturing them with M-SCF 10 ng/ml (574804, BioLegend) for six days and polarizing them for one day with IFN-γ 20 ng/ml (285-IF-100, R&D Systems) or IL-4 20 ng/ml (200-04,

PeproTech), respectively. Control cells (M0) were cultured with M-CSF for seven days. Commercially available primary human lymphatic endothelial cells (HLEC) isolated from lymph nodes (Catalog #2500, ScienCell Research Laboratories) or human dermal lymphatic endothelial cells (HDLEC) isolated from skin (C-12216, PromoCell) were cultured in the appropriate culture media supplemented with serum and antibiotics. Cells were harvested and subjected for RNA extraction.

Stromal cells were isolated from peripheral and mesenteric lymph nodes of WT mice with enzymatic digestion and cultured for 7 days in Complete mouse endothelial cell medium /w Kit (M1168, Cell Biologics). LECs were enriched with cell sorting, cultured for a further 7 days and used for performing adhesion assays.

Table 5. Antibodies against human antigens used in microscopy and adhesion assays.

ANTIBODY	CLONE	CAT. NO.	COMPANY	PUB.
Clever-1	3-372	Irjala *et al.*, 2003	In-house	II
IgG Fc-biotin	pAb	ab98561	Abcam	I
Msr1	pAb	ab123946	Abcam	I
LYVE1	pAb	103-PA50	ReliaTech GmbH	II
Prox-1	pAb	AF2727	R&D Systems	II
Robo4	pAb	NB110-58780	Novus Biologicals	II
Robo4	pAb	ab10547	Abcam	II
Siglec-1	HSn 7D2	ab18619	Abcam	I
Streptavidin AF488	-	S11223	Invitrogen	I

4.9.4 RT-PCR and qPCR (I, II)

RNA was extracted from human macrophages (M0, M1 and M2), cultured primary human LECs and murine B cells isolated from Peyer's patches with the B cell Isolation Kit (130-090-682, Miltenyi Biotec). RNA extraction was performed using the NucleoSpin RNA extraction kit (740955.250, Macherey-Nagel) and RNA was transcribed to complementary DNA with the SuperScript VILO cDNA Synthesis kit (11754050, Invitrogen). RT-PCR reactions were conducted with Kapa2G Robust PCR Kit (Kapa Biosystems) and quantitative PCR (qPCR) was performed using the TaqMan Universal Master Mix II (4440038, Thermo Fisher Scientific) with probes and primers designed with the Universal Probe Library Assay Design Center (Roche Life Science, Table 6). qPCR reactions were ran with the Applied Biosystems' Quant Studio 3 Real-Time PCR System (Thermo Fisher Scientific) and the Applied Biosystem's analysis modules available in the Termo Fisher Cloud computing platform were used to calculate relative expression levels. Results are

presented as the percentage of control gene (*Actb*) mRNA levels from the same sample.

Table 6. Primers used for RT-PCR and qPCR.

GENE	PRIMER	SEQUENCE	PROBE	PUB.
Actb (human)	Left Right	tgacggggtcacccacactgtgcccatcta ctagaagcatttgcggtggacgatggaggg	-	I
Actb (mouse)	Left Right	ctaaggccaaccgtgaaaag accagaggcatacagggaca	64	II
Msr1 (human), variants 1 and 3	Left Right	caggtccaataggtcctccggg (F1) gagcagcgatttcatagttgtg (R1)	-	I
Msr1 (human), variant 2	Left Right	caggtccaataggtcctccggg (F1) gtatgagcatgggagcagaggc (R2)	-	I
Cxcr4 (mouse)	Left Right	tggaaccgatcagtgtgagt gggcaggaagatcctattga	38	II
Cxcr5 (mouse)	Left Right	gaatgacgacagaggttcctg gcccaggttggcttcttat	13	II
S1pr1 (mouse)	Left Right	cggtgtagacccagagtcct agcttttccttggctggag	66	II

4.10 Statistical analyses (I–III)

Experimental sample sizes were determined using pilot assays and experiments were conducted in a non-blinded fashion. The numerical data is presented as mean ± SEM or mean ± SD. For comparing results between two treatment groups or genotypes, data was analyzed using the Student's t test, Mann-Whitney *U* test or the Wilcoxon matched-pairs signed-rank test. For comparisons between multiple groups, data was analyzed with the Kruskal-Wallis test followed by Dunn's test when applicable. To analyze fluorescence intensities in different zones of the lymph node, linear regression analyses were applied to the data. Statistical analyses were conducted with GraphPad Prism or SAS 9.4 software. *P* values under 0.05 were considered as statistically significant.

5 Results

5.1 Differentially expressed genes in lymph node afferent and efferent LECs (I)

Molecular differences of the lymph node afferent and efferent LECs have not been thoroughly studied before. Therefore, we set out to analyze the genetic differences among these populations. LECs were isolated from lymph nodes by using fluorescence-activated cell sorting and laser capture microdissection. LECs were sorted as Live/Lineage$^-$ podoplanin$^+$ cells and they were further divided into afferent and efferent populations based on expression of CD73, a marker for the afferent lymphatics (Ålgars et al., 2011).. For laser capture microdissection, LECs were distinguished by their expression of CD31, while F4/80 was used to label lymph node macrophages. Cells were excized from the SCS and cortical sinus areas representing the afferent and efferent lymphatics, respectively. The cortical sinus was selected over the medullary sinus, as lymphocytes primarily enter the cortical sinus during egress. RNA from the isolated cells was subjected to a whole-genome wide microarray to reveal differentially expressed genes among the analyzed populations. The results contained a large number of genes that were predominantly expressed by the afferent or efferent cell population. These genes encoded several different families of molecules, such as chemokines, integrins and other regulators of inflammatory pathways. Among the results, Msr1 and Robo4 were predominantly expressed in the afferent and efferent cell populations, respectively. Their expression was validated at the protein level and their role in the lymphatics was examined with further studies. (I: Fig. 1a-c, SFig. 1-3, Table S1-2)

5.2 Msr1 is expressed by SCS LECs (I)

Msr1 is a transmembrane protein that belongs to the Class A scavenger receptors. It is predominantly expressed in anti-inflammatory type 2 macrophages, where it recognizes modified self and foreign molecules and is involved in their removal. Numerous ligands have been reported for Msr1, the most prominent being acetylated low-density lipoprotein. (Canton et al., 2013; Kelley et al., 2014) Our

microarray revealed Msr1 to be preferentially expressed by the afferent LECs in comparison to efferent LECs. Image analysis showed Msr1 staining in the SCS floor LECs, while Msr1 could not be detected in cortical sinuses. The lymph node medullary sinus stained positively for Msr1; however, the staining could derive from both LECs as well as macrophages, in theory. Similar staining patterns for Msr1 were detected in the MLN, while Peyer's patches devoid of afferent lymphatics were negative for Msr1. Notably, SSM do not express Msr1 and therefore, Msr1 expression in the afferent LECs is not derived from contaminating macrophages in the samples subjected for microarray. (I: Fig. 1d, Fig. 2-3, SFig. 4) We wanted to investigate whether $Msr1^+$ afferent lymphatics would be present in the periphery and analyzed skin lymphatics with flow cytometry and imaging. Msr1 expression in LECs of the skin can be detected, albeit at very low levels. (I: Fig. 4, SFig. 5) As this was the first time Msr1 expression has been described in LECs, we investigated whether they express the same isoforms of Msr1 as macrophages. By analyzing human primary LECs isolated from the skin and lymph node as well as monocyte derived macrophages, we were able to detect transcripts of isoforms 1 and 2, but not isoform 3, for Msr1 in both cell types. (I: SFig. 7)

5.3 Msr1 regulates lymphocyte migration in the SCS (I)

During migration to the lymph node via lymphatic vessels, lymphocytes must adhere to endothelial cells in the periphery, transmigrate through the LEC layer, migrate along the lymphatic vessels and finally, adhere and transmigrate through the SCS LEC floor. To study whether Msr1 plays any role in regulating cell adhesion, we performed adhesion assays with mouse and human frozen lymph node sections as well as primary mouse LECs. Lymph nodes were isolated from Msr1 KO, WT mice or humans, and frozen sections were treated with blocking antibodies against Msr1 or their isotype controls. Isolated lymphocytes were let to bind to the sections and adhered lymphocytes were assessed by microscopy. For these experiments, SSMs were depleted from mouse lymph nodes with s.c. injections of clodronate containing liposomes to remove the potential effect of macrophages on lymphocyte adhesion. In similar experiments, adherence of lymphocytes to primary mouse LECs treated with an Msr1-targeting blocking antibody or its isotype control was determined. In all cases, binding of lymphocytes to the SCS region of lymph nodes, or directly to LECs, was reduced by 60% when Msr1-mediated binding was abrogated. Direct binding of Msr1 to $B220^+$ B cells, $CD4^+$ T cells as well as $CD11b^+$ myeloid cells, but not $CD8^+$ T cells, was detected using an Msr1-Fc fusion protein. (I: Fig. 5b-f, i, SFig. 5a-b)

To analyze the role of Msr1 in regulating lymphocyte migration, we performed *in vivo* experiments utilizing Msr1 KO animals. Analyzing lymph nodes from Msr1 KO mice revealed them to have a comparable lymphocyte composition in comparison to their WT controls. Similarly, the lymph node lymphatic and blood vasculature appeared normal. (I: Fig. 5a) To study the role of Msr1 in lymphocyte migration via the lymphatics, we adoptively transferred CFSE-labeled lymphocytes s.c. into Msr1 KO and WT recipient animals and analyzed the migrated lymphocytes that were recovered from draining popliteal lymph nodes with flow cytometry and fluorescence imaging. Lymphocyte migration appeared normal in Msr1 KO animals when analyzing migrated cells 12 hours post adoptive transfer. When analyzing the distance of the migrated cells from the SCS at 4.5 hours after transfer, lymphocytes in Msr1 KO recipients migrated 30% further into the draining lymph node parenchyma in comparison to WT animals. This suggests that Msr1 may have a role in promoting retention of lymphocytes in the SCS through direct adhesion. (I: Fig. 5g-h, SFig. c-f)

5.4 Robo4 is expressed in efferent lymphatics (II)

Robo4 was selected as a target molecule predominantly expressed by the efferent lymphatics. Originally, Robo4 was discovered to be expressed in vascular endothelial cells (Huminiecki et al., 2002; Okada et al., 2007) and later in primary human LECs (Yu et al., 2014). Robo4 belongs to the family of neuronal guidance molecules, the roundabout proteins, which consist of four different receptors, Robo1-4 (Legg et al., 2008). It has a role in inhibiting angiogenesis and endothelial permeability (Jones et al., 2008) as well as in regulating stem cell migration (Smith-Berdan et al., 2011, 2015). We studied expression of Robo4 in the lymphatics with flow cytometry and fluorescence imaging and were able to detect protein level expression of Robo4 in LEC and BEC populations isolated from peripheral lymph nodes, MLNs and Peyer's patches. To image Robo4 expression in the lymph node, we produced a monoclonal antibody against murine Robo4 by taking advantage of the Robo4 deficient mouse. The generated monoclonal antibody was administered s.c. into the footpads of WT mice to label Robo4$^+$ cells in the draining lymph node *in vivo*. Imaging data revealed Robo4 to be located in the medullary sinus of the draining lymph node while the SCS LECs were mostly devoid of any signal. Robo4 was similarly expressed in human lymph node blood vessels and lymphatic sinus LECs as well as in the lymphatic vasculature of the appendix, which were observed by traditional immunofluorescence stainings *in vitro*. (II: Fig. 1-2, SFig. 1-2)

To study the role of Robo4 in efferent lymphatics we concentrated our further studies to the Peyer's patch as it is devoid of afferent lymphatics. Analyses of the

proportion and distribution of main lymphocyte subpopulations within the Peyer's patch proved to be comparable between Robo4 KO and WT mice. In comparison, the amount of B cells was diminished in peripheral lymph nodes, as was the actual number of T cells. The lymphatic vasculature, blood vessels and MAdCAM-1$^+$ HEVs appeared normal in Robo4 KO Peyer's patches. (II: Fig. 3, SFig. 3)

5.5 Robo4 contributes to the turnover of Peyer's patch naïve B cells (II)

Similar to Msr1, Robo4 regulates lymphocyte adhesion to the lymphatic sinus of human lymph nodes, which were pretreated with antibodies against Robo4 or their respective controls. We examined the role of vascular Robo4 in regulating entry of lymphocytes into lymph nodes and found Robo4 to regulate the early entry of B cells into Peyer's patches and the MLN as well as the entry of T cells into peripheral lymph nodes and MLN at a later timepoint. A concomitant increase of adoptively transferred lymphocytes was detected in the peripheral blood of Robo4 KO mice (2-hour timepoint), whereas the percentage of transferred cells was found to be reduced later (18-hour timepoint). (II: Fig. 2d, 4, SFig. 4-6) To study the turnover of Peyer's patch lymphocytes, they were labeled *in situ* with FITC, and the number of lymphocytes remaining in the Peyer's patches was examined 24 hours after labeling. These migration experiments revealed that the percentage of naïve B cells retained in Robo4 KO mice was enlargened in comparison to their WT controls. The endogenous CD19$^+$CD62L$^+$CD44$^-$ naïve B cells reside in the follicle surrounding GCs in the Peyer's patch. B cell development in the bone marrow was not affected by depletion of Robo4 and analysis of different B cell subpopulations recovered from Peyer's patches revealed no differences among B cells in Robo4 KO and their WT controls. Adoptive transfer of lymphocytes into the footpads of Robo4 KO mice and their controls suggested that lymphocyte egress might be hindered in the absence of Robo4. Interestingly, $S1P_1$ known to regulate B cell egress in the Peyer's patch was expressed less at the mRNA level in Robo4 KO B cells. Altogether, these results suggest that Robo4 contributes to the turnover of Peyer's patch B cells. (II: Fig. 5, SFig. 7-9)

5.6 Robo4 and gut lymphoid tissue homeostasis (II)

We reasoned that the increased amount of retained naïve B cells in Robo4 KO Peyer's patches would cause the tissues to grow unless their cell number would be kept constant with complementary apoptosis. We examined the rate of apoptosis and discovered that the percentage and number of Annexin V$^+$ early apoptotic cells

was slightly increased in Robo4 KO Peyer's patches. In contrast, end-phase apoptotic cells with fragmented DNA detected with the TUNEL staining kit were not increased. (II: Fig. 6) Therefore, increased naïve B cell turnover in Robo4 KO mice is not markedly compensated for by higher rates of apoptosis. This suggests that the size of Robo4 KO Peyer's patches may gradually increase over time unless other factors regulate their cell turnover.

Examining the tissue weights of Robo4 KO Peyer's patches and MLNs revealed that the mass of MLNs increases in Robo4 KO mice over time. Although expected, Peyer's patches did not show relative increase in their weight. To study what might cause growth of MLNs we analyzed different lymphocyte populations but found no single population to be drastically increased. Analysis of apoptotic cells revealed that in fact, Robo4 KO mice exhibit more dead cells among lymphocytes in their MLNs. As hematopoietic cells did not offer any explanation to the phenomenon, we examined the stromal compartment. The largest population of stromal cells is the FRCs that ensheath the collagen-rich reticular conduit. Stainings and image quantification of collagen I revealed a marked increase in collagen I^+ staining in the MLNs of Robo4 KO mice, which may offer a reasonable explanation for the observed increase in tissue weight. (II: Fig. 7)

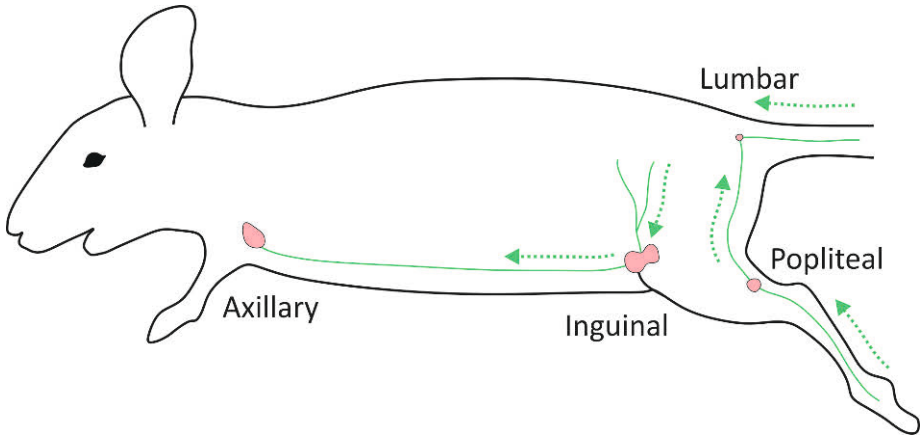

Figure 6. Lymph nodes and their draining patterns relevant to study III. Antibodies and other *in vivo* administered reagents were injected subcutaneously (s.c.) into the footpads of recipient mice. Entry of antibodies into the draining (popliteal) and non-draining (contralateral popliteal, inguinal or axillary) lymph nodes were examined. In some cases, the second lymph node in the chain (lumbar, also known as sacral) was analyzed. Arrows indicate the direction of lymphatic drainage. Figure adapted from Kawashima *et al.*, 1964.

5.7 Transfer of antibodies into the draining lymph node parenchyma (III)

The prevailing dogma has been that small soluble antigens under 70 kDa enter the lymph node conduit, whereas large antigens over 70 kDa are transported to lymph nodes only within migratory DCs (Mueller and Germain, 2009). We detected that a pool of s.c. administered fluorescently conjugated antibodies (size 150 kDa) against B cells (B220), helper T cells (CD4), endothelial cells (CD31) and DCs (CD11c) were instantly and locally transferred into the parenchyma of draining lymph nodes. Antibodies remained intact and were able to bind their native epitopes, which was detected by flow cytometry and fluorescence imaging. The administered antibodies could be detected only from the single draining lymph node (popliteal), or the next node along the chain (lumbar, Figure 6). Antibodies were not present in any non-draining lymph nodes (inguinal, axillary) highlighting the function of the peripheral lymphatic vessels, which transported the antibodies to the draining lymph node. Experimentally administered antibodies entered the lymph node cortex and paracortex readily, however, their penetration into B cell follicles was not complete, nor were they able to reach the lumen of HEVs. (III: Fig. 1, SFig. 1)

The transfer of antibodies from the SCS into the parenchyma occurred in a dose-dependent manner. Transfer was extremely efficient and antibodies could be detected in draining lymph nodes 30 seconds after having been administered. Antibody transfer was independent of the target antigen, host or fluorochrome. However, the size of antibodies limited their entry into the lymph node as IgM antibodies (size 900 kDa) were excluded from the parenchyma. (III: Fig. 2, SFig. 3)

5.8 Antigens are transported in fluid phase (III)

To begin dissecting the mechanism regulating the transport of antibodies across the SCS, we examined whether neonatal Fc receptor (FcRn, *Fcgrt*) known to transport IgG antibodies across the placenta and neonatal gut epithelium (Pyzik et al., 2015) would regulate transport. According to data generated by the Immunological Genome Project, *Fcgrt* is abundantly expressed in LECs. However, s.c. delivered antibodies were transported into the draining lymph nodes of FcRn KO mice as efficiently as into their WT controls. Blocking FcRIIb (*Fcgr2b*) with a function-blocking antibody did not inhibit transfer of antibodies. Transport was also not regulated by the Fc tail of antibodies, as its deglycosylation or absence did not affect the outcome. (III: Fig. 3a–b, d, SFig. 4a–d, f) Complement receptors had no role in transporting antibodies since s.c. administered IgA and IgY, which is not bound by any mammalian Fc or complement receptor, were able to enter the

parenchyma. To investigate whether antibody transport is dependent on receptors, we s.c. delivered the pool of test antibodies in the presence of competing antibodies. These experiments revealed antibody transport across the SCS to be receptor independent. (III: Fig. 3c, e, SFig. 4e, g-i)

Fluorescence imaging of an *in situ* fixed, nonbinding unconjugated rat IgG2a antibody (referred to as free antibody) revealed that the transferred antibody colocalized with the SCS floor LECs. Moreover, it was detectable between lymphocytes of the lymph node parenchyma suggesting the antibody to float freely in the interstitium. The free antibody created a centrifugal concentration gradient with the highest concentration directly under the SCS. Similarly, s.c. administered fluorescently labeled dextrans (70 and 500 kDa in size) were transferred into the lymph node parenchyma, displayed a concentration gradient and surrounded parenchymal lymphocytes. These results indicated that antibodies and other model antigens are transported across the SCS floor in fluid-phase. (III: Fig. 4)

5.9 LECs transcytose antigens across the SCS (III)

The known mechanisms for antigen transport from the SCS to the lymph node parenchyma are the reticular conduit and SSMs, which neither regulated transcytosis of antibodies. (III: Fig. 5–6) Endothelial cells are known to actively transport macromolecules from one surface to the other using transcellular vesicular transport, i.e. transcytosis, which has been reported in BECs (Fung et al., 2018; Yazdani et al., 2019) and LECs in the intestine and skin (Dobbins and Rollins, 1970; Triacca et al., 2017). We asked whether SCS LECs would transport lymph-borne antibodies and observed LECs to be rich in heterogenous vesicular structures determined with electron microscopy. The lymph-borne free antibody localized within these vesicles of the SCS LECs while intercellular junctions were devoid of the antibody. High-resolution imaging demonstrated free antibody vesicles in the $CD31^+$ SCS floor LECs, where they localized within or in direct contact with endosomes expressing the early endosomal marker EEA1. (III: Fig. 7, SFig. 5, Supplemental Video 1)

Endocytosis involves the generation of small vesicular structures formed from the plasma membrane, which transport cargo into the cell cytoplasm. Known pathways of endocytosis are macropinocytosis, clathrin-dependent, caveolin-dependent and clathrin-independent endocytosis. Dynamin is required for the releasing of the endocytic vesicle from the plasma membrane. (Doherty and McMahon, 2009; Ferreira and Boucrot, 2018; Kaksonen and Roux, 2018) To describe the mechanism regulating transcytosis of antibodies, we experimentally manipulated the known endocytic pathways. The pool of test antibodies was s.c. administered into Cav1 KO mice, where we observed intact transport of antibodies

into the lymph node parenchyma. Chemical inhibitors disrupting clathrin-mediated endocytosis (Pitstop 2, monodansylcadaverine), macropinocytosis (EIPA, imipramine) and function of dynamin (Dyngo-4a, Dynole 34-2) were administered topically *in vivo* on surgically exposed lymph nodes to block the respective pathways. In addition, intercellular junctions were stabilized with the topical administration of adrenomedullin *in vivo*. After inhibition, the pool of test antibodies was delivered s.c. and the entry of antibodies into the lymph node parenchyma was detected with flow cytometry or fluorescent imaging. No abrogation of antibody transport was observed, except after the administration of the dynamin blocking inhibitors Dyngo-4a and Dynole 34-2. Our results describe LECs as the cells mediating active dynamin-dependent, fluid-phase transcytosis of antibodies across the SCS into the lymph node parenchyma. (III: Fig. 8–9, SFig. 6)

5.10 Applications for s.c. administered antibodies (III)

We reasoned that LEC-mediated transcytosis would transport other protein antigens into the lymph node similar to antibodies. Indeed, an s.c. delivered human protein was detected 2 hours after injection in lymph node resident DCs and macrophages, suggesting that immune reactions can start immediately after antigens get access to the lymph node parenchyma. (III: Fig. 10) We were also able to locally activate T cells in the draining lymph node by s.c. administering antibodies binding to the T cell receptor and CD3, and by detecting the increased amount of T cell activation markers CD69 and CD25. T cell activation was also examined using an adoptive transfer model, where naïve $CD45.2^+$ $CD4^+$ T cells expressing the transgenic T cell receptor for OVA (OTII cells) were isolated, labeled with CFSE and introduced intravenously into $CD45.1^+$ congenic recipients. OVA was administered with a T cell activating (clone D665) or inhibiting (clone 37.51) anti-CD28 antibody, a negative IgG or positive LPS control. Three days later the draining popliteal lymph nodes were isolated and the proliferation of T cells was examined with flow cytometry. When $OTII^+$ T cells were activated with clone D665, they proliferated specifically in the draining lymph node, whereas administration of the CD28 inhibiting clone 37.51 reduced the proliferation of OVA-specific T cells. Finally, the activating (clone D665) and inhibiting (clone 37.51) anti-CD28 antibodies were s.c. administered into different legs of the same recipient animal, where they exerted the same local activating and inhibiting functions. (III: Fig. 11b–g, SFig. 7d–e)

Whole-organ imaging of optically cleared tissues reveals a tremendous amount of information on tissue anatomy. However, sample preparation is time consuming and usually requires overnight staining of each primary and secondary antibody.

(Susaki and Ueda, 2016) We sought out to test whether the transport of lymph-borne antibodies into the draining lymph node parenchyma could be utilized to improve whole mount imaging. The pool of test antibodies against B cells (B220), helper T cells (CD4), endothelial cells (CD31) and DCs (CD11c), was administered s.c. and the draining lymph nodes were harvested after 30 min. Samples were lightly fixed, optically cleared using a mixture of benzyl alcohol and benzyl benzoate (BABB), and imaged immediately with a confocal microscope. Imaging the lymph nodes revealed that LEC mediated transcytosis can be harnessed to expedite the preparation of whole mount samples reducing sample preparation from days to a couple of hours. (III: Fig. 11a, Supplemental video 2)

6 Discussion

6.1 Heterogeneity of lymph node LECs (I)

The lymph node contains LECs at its sinuses, where they are in direct contact with migrating cells and lymph contents. We analyzed the molecular differences of the afferent and efferent LECs in the lymph node SCS and lymphatic sinus. The determined cell populations were isolated with laser capture microdissection and cell sorting and subjected to a genome-wide microarray. The methods and markers used for isolating the samples did not yield pure LEC populations. Namely, cells were sorted based on positive expression of podoplanin without the endothelial marker CD31 or exclusion of FRCs. Laser capture microdissection, like any method, also has its limitations. Most likely, the cell samples isolated by manual excision contain material from other SCS cells, such as macrophages and lymphocytes. Therefore, combining the two isolation methods is essential, as is the thorough validation of the hit molecules.

Heterogeneity of immune cells is under heavy investigation. Many of the lymph node cell populations, such as FRCs, have been subjected for microarray analyses and published as part of the Immunological Genome Project (Malhotra et al., 2012). Genome-wide microarrays have been followed by deep sequencing techniques such as RNA sequencing, which is able to detect previously unknown genes as well as genetic polymorphisms and isoforms (Malone and Oliver, 2011). Currently, single-cell sequencing has emerged as a leading-edge technique allowing unbiased analysis of samples, where the analyzed cells are clustered according to their transcriptome. Although the method is highly sensitive and reveals a great amount of new genetic information, single-cell sequencing does not yield spatial information of the analyzed genes. In addition, it cannot be used for detecting non-coding or bacterial RNA. (Papalexi and Satija, 2017) Recently, two studies reporting single-cell sequencing of the mouse lymph node LECs were published. Lymph node LECs were reported to cluster into valve, SCS ceiling, SCS floor and two separate clusters of medullary sinus LECs. (Fujimoto et al., 2020; Xiang et al., 2020) Human lymph node LEC heterogeneity was also recently reported (Takeda et al., 2019). Our research compared the differentially expressed genes between two LEC populations using a more conventional microarray. These

results can now be examined and compared to data generated by single-cell sequencing to validate our findings.

6.2 Msr1 and Robo4 – newly described lymphatic markers (I, II)

Based on our microarray results, we selected Msr1 and Robo4 for functional studies. Msr1 is a scavenger receptor originally reported to be expressed in macrophages, where one of its functions is to recognize and remove pathogens and other foreign substances (Kelley et al., 2014). We discovered Msr1 to be expressed in the SCS LECs, while cortical sinus LECs were negative. Interestingly the medullary sinus stains positive for Msr1. As the medullary sinus contains LECs as well as macrophages and DCs, which may also express Msr1, flow cytometry analysis of medullary sinus LECs can verify whether they truly express Msr1. Based on single-cell sequencing, medullary sinus LECs cluster as a separate population from cortical sinus LECs (Fujimoto et al., 2020; Xiang et al., 2020). Therefore, it may well be possible that Msr1 expression is differently regulated by LECs of the cortical and medullary sinus.

Robo4 expression in the lymphatics was validated using flow cytometry and fluorescence imaging with an in-house antibody. Peripheral lymph nodes and the MLN displayed heterogeneous expression of Robo4 among LECs, whereas Robo4 was homogeneously expressed in Peyer's patch lymphatics. In humans, Robo4 is highly expressed in the vasculature of several solid tumors, such as melanoma, renal, lung and liver cancer, while it is absent from normal tissue. Most likely Robo4 expression is induced by low shear stress, which would explain why healthy blood vessels do not express Robo4. (Mura et al., 2012; Seth et al., 2005) Due to its expression in tumor tissue, Robo4 has been studied as a drug target. Vaccinations against Robo4 reduce tumor growth in lung carcinoma (Zhuang et al., 2014) and Robo4 can be utilized for the delivery of anti-tumor therapy using Robo4-detecting antibodies (Yoshikawa et al., 2013). Robo4 is also expressed in several subpopulations of human lymph node LECs (Takeda et al., 2019). Therefore, anti-tumor therapy utilizing Robo4 as an address molecule may guide cancer drugs to lymph nodes, where they could be available to reduce tumor metastases. Alternatively, the delivery of tumor cell attacking drugs into lymph nodes could give rise to adverse effects.

6.3 Msr1 and Robo4 regulate lymphocyte migration (I, II)

Cell migration across the SCS is known to be regulated by several molecules (Martens et al., 2020; Rantakari et al., 2015; Ulvmar et al., 2014). We discovered that Msr1 is another regulator of lymphocyte entry from the SCS into the lymph node parenchyma. In the absence of Msr1, lymphocytes migrated significantly further into the lymph node parenchyma 4.5 hours after their adoptive transfer, when compared to their controls. This suggests that Msr1 may function as a retaining molecule in SCS LECs regulating the early migration of lymphocytes through their adhesion to LECs. We also observed adoptively transferred WT lymphocytes in the medullary sinus of the lymph node, as reported by others (Braun et al., 2011). Msr1 expressed in the medullary sinus may take part in regulating lymphocyte migration into the lymph node parenchyma. In the case of Robo4, we detected it to regulate lymphocyte entry via HEVs to examined lymphoid tissues as well as the turnover of naïve B cells in Peyer's patches. As Msr1 and Robo4 can both mediate direct binding of lymphocytes to the lymph node sinuses, they may directly have a role in regulating lymphocyte migration by providing retention of lymphocytes. An indirect role via other receptors or cell types can also be the underlying mechanism.

We discovered little differences in the lymphocyte compositions of the peripheral lymph nodes of Msr1 and Robo4 KO mice at homeostatic conditions. The fact that these mice appeared rather normal suggests that lymphocyte migration into lymph nodes is not greatly disrupted. As a drastic comparison, *plt/plt* mice, which have a naturally occurring mutation resulting in the complete lack of CCL19 and CCL21, have a very small count of T cells in their lymph nodes (Nakano et al., 1997). All of our studies were performed using full KO mice with the genes of interest depleted in all cells and throughout the whole lifespan of the animal. Hence, it is plausible that compensatory mechanisms arise in the KO animals, which may mask the effect of Msr1 or Robo4. Utilizing conditional knockout animals created with the Cre-loxP system would specifically allow investigating the role of Msr1 and Robo4 solely in LECs. As Msr1 is expressed by LECs as well as cells of hematopoietic origin, another approach would be creating bone marrow chimeras by reconstituting WT recipients with Msr1 KO bone marrows allowing for the analysis of LEC specific Msr1.

Lymphocyte migration involves both the migrating cell and its surrounding tissue. In most of our adoptive transfer experiments, we transferred WT cells into Msr1 or Robo4 deficient host animals. However, when studying the turnover of B cells in Robo4 KO Peyer's patches, we were in fact studying the turnover of endogenous B cells in their natural environment. Robo4 is expressed by hematopoietic stem cells in the bone marrow, and its mRNA expression decreases

during lymphocyte maturation (Smith-Berdan et al., 2011). While lymphocytes do not express Robo4 mRNA, the effects of its expression during the stem cell phase may regulate other molecules. We studied mRNA expression of *Cxcr4, Cxcr5* and *S1pr1* in Peyer's patch B cells. All these genes have been reported to be important in regulating lymphocyte egress in the Peyer's patch (Pham et al., 2010; Schmidt et al., 2013). As an unexpected finding, the level of *S1pr1* expression was diminished in Robo4 KO mice. Therefore, changes in the gene signature of the migrating lymphocytes in Robo4 KO animals may contribute to the impairment in the detected B cell turnover.

During inflammation, the lymph node faces drastic changes as LECs and other stromal cells undergo massive expansion to facilitate the increased number of proliferating lymphocytes. In addition, lymphocyte migration into lymph nodes is increased; lymphocyte egress is shut down and the molecular composition of endothelial cells changes. (Thierry et al., 2019) Studies of Msr1 and Robo4 as regulators of lymphocyte migration were conducted under homeostasis and their possible role during inflammation remains to be analyzed. However, Msr1 may well exert scavenging functions in endothelial cells, which has been reported for another scavenger receptor, CD36 (Huang et al., 2019). Endothelial Robo4 has been studied during inflammation, where it has anti-inflammatory properties (Zhao et al., 2014), it suppresses endothelial hyperpermeability (Shirakura et al., 2019) and regulates cellular crosstalk between endothelial cells and monocytes (Shirakura et al., 2017). Similarly, Robo4 may have a more marked role in regulating lymphocyte migration or other LEC-mediated functions during inflammatory conditions.

6.4 Robo4 contributes to MLN homeostasis (II)

When studying the turnover of B cells in Robo4 KO mice, we reasoned that the observed increase of B cells would eventually result in larger Peyer's patches if not compensated for by apoptosis. We observed a small increase in the number of early apoptotic cells (Annexin V^+) among $CD45^+$ hematopoietic cells, yet, the number of late apoptotic cells was not increased based on the TUNEL assay, which detects fragmented DNA. This discrepancy may be due to the different methods used for detection. The whole number of apoptotic cells can be analyzed easily with flow cytometry, whereas imaging only concentrates on a single plane of the tissue. It is also possible that apoptosis may be differentially regulated in Robo4 KO mice, and our methods did not detect this highly dynamic process.

Unexpectedly, we observed a change in the size of MLNs in Robo4 KO mice. There was a significant increase in tissue growth among adult Robo4 KO mice (>12 weeks of age) in comparison to their WT controls, and we sought out to

investigate what might cause this prominent 20% increase in tissue weight. No differences explaining the phenomenon were detected among the studied immune cells in the MLNs. Therefore, we investigated whether there were any changes in the stroma and concentrated on FRCs, which are the most abundant stromal cells. To visualize the conduit ensheathed by FRCs, we stained and quantified collagen I^+ signal in MLNs of Robo4 KO and their WT counterparts. Surprisingly, the amount of collagen I was markedly increased in the MLNs of Robo4 KO mice suggesting an increase in the amount of FRCs or in their synthesis of collagen. Robo4 is not expressed in FRCs according to Immunological Genome Project data, nor could we detect $Robo4^+$ FRCs in the peripheral lymph node. Hence, Robo4 on other cell types may indirectly regulate the tissue environment in the MLN favoring an increased deposition of collagen. Indeed, crosstalk between different stromal cell compartments or between stromal cells and immune cells has been previously reported. FRCs are known to regulate vascular cell permeability via production of VEGF (Chyou et al., 2008) and they may also regulate production of ECM molecules in response to contact with lymphocytes (Katakai et al., 2004).

6.5 Large antigens are transported into the lymph node parenchyma (III)

The filtering function of the lymph node has been well documented in plenary publications, which also determined the size limit of soluble molecules physically able to enter the reticular conduit (Gretz et al., 2000; Sixt et al., 2005). Challenging the widely accepted limits of size-selective filtering at the SCS, we discovered that large lymph-borne antigens with a size at least up to 500 kDa could enter the lymph node parenchyma. Resident DCs have been shown to sample the conduit content and directly acquire antigens from there (Sixt et al., 2005). However, transcytosed antigens are also available for DCs directly in the lymph node parenchyma. As lymph is full of proteins derived from the organs it drains (Clement and Santambrogio, 2013) as well as endogenous antibodies (Leak et al., 2004; Worm, 1981), all of these molecules are instantly drained from the periphery into the lymph node, where they are available for immune cells.

Although the filtering function of the lymph node has been thoroughly documented, the presence of parenchymal antibodies or other large antigens has not previously been reported. Parenchymally transported antibodies and dextrans can easily remain under the detection limit if not specifically studied. Transport of antigens into the lymph node parenchyma has been observed before, although the doses of administered antibodies have been large and transport was observed over several minutes to hours in comparison to our observations in 30 seconds. Moreover, no molecular mechanism describing the phenomenon was reported.

(Gerner et al., 2017; Hauser et al., 2007; Pape et al., 2007; Roozendaal et al., 2009) Combining imaging with flow cytometry allowed us to directly verify the binding of s.c. administered antibodies to lymph node parenchymal lymphocytes confirming our results. Moreover, we were able to physically visualize the free antibody within SCS floor LECs, where it colocalized with $EEA1^+$ endosomes. Whether transport of antigens and antibodies increases during inflammation remains to be studied. *In vitro* studies have reported that transcytosis in peripheral LECs increases during flow and inflammatory stimuli (Triacca et al., 2017), therefore inflammation-induced changes may also increase the levels of large antigen or antibody transcytosis in the lymph node.

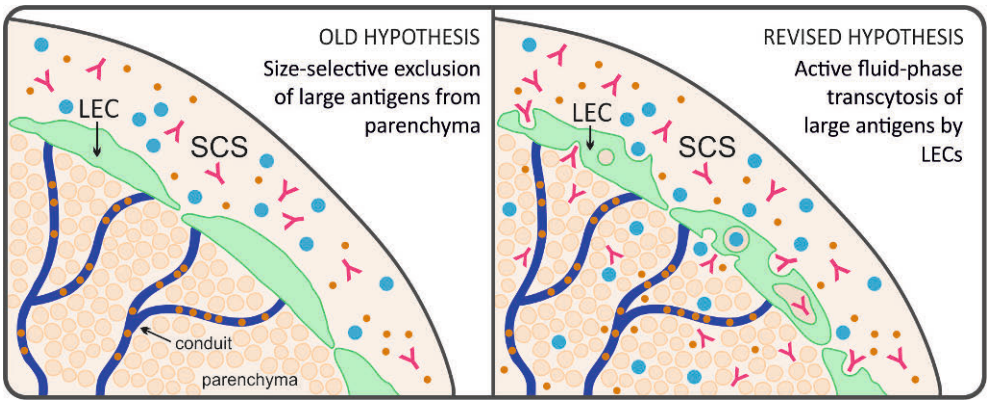

Figure 7. Mechanism for the transfer of large lymph-borne soluble antibodies across the subcapsular sinus (SCS). Lymphatic endothelial cells (LEC) actively transcytose antibodies and other antigens from the SCS lumen to the lymph node parenchyma. Fluid-phase transcytosis is dynamin dependent and most likely resembles ultrafast endocytosis independent of other described mechanisms of endocytosis.

6.6 Active vesicular transcytosis in LECs (III)

LECs transport lymph-borne antibodies across the SCS by fluid-phase dynamin-dependent transcytosis (Figure 7). If LECs were once viewed as just endothelial cells passively lining lymph node sinuses, recent research has highlighted them as a multifunctional cell population of the lymph node (Jalkanen and Salmi, 2020). Endocytosis in endothelial cells was first described for BECs. Yet, early electron microscopy studies already recognized vesicular structures in LECs of intestinal villi, where vesicles accounted for 15% of the cytoplasm volume and transported water-soluble molecules (Dobbins and Rollins, 1970). Peripheral afferent LECs have been reported to endocytose albumin and 70 kDa dextrans. This vesicular transport plays a significant role in transporting fluids and is mediated by dynamin-dependent, clathrin- and caveolae-mediated endocytosis. (Triacca et al., 2017)

We observed lymph node LECs to be rich in vesicular structures in agreement with reports from other organs. The mechanism of LEC-mediated transcytosis was determined as dynamin-dependent, caveolin and clathrin-independent endocytosis as a sum of several observations. The role of caveolae was excluded, as antibody transport was intact in Cav1 KO mice. Receptor-independence of antibody transport suggests that clathrin, which is known to be triggered by receptor ligation, is not involved. In addition, the formation of clathrin-coated vesicles takes up to 30-120 seconds. Macropinocytosis also requires receptor activation and should be visible due to the large size of macropinocytic vesicles (0.2-10 μm). (Kaksonen and Roux, 2018) We interfered with the different endocytic pathways by utilizing chemical inhibitors. All results combined, our data strongly suggests LEC-mediated transcytosis to be a previously unknown, extremely fast mechanism of transport, which resembles ultrafast clathrin-independent endocytosis (Watanabe and Boucrot, 2017). Antibodies are most likely not excluded from other vesicles and therefore, may also be endocytosed passively by other pathways.

The SCS floor is formed by LECs located on top of the ECM, macrophages and DCs. The discontinuous ECM of the SCS floor was thought to allow diffusion of antigens into the lymph node parenchyma over time (Pape et al., 2007), although they were most likely transcytosed into the parenchyma by LECs. In theory, antibodies might also get access into the lymph node parenchyma through intercellular junctions. Our analyses revealed intercellular junctions to be devoid of the free antibody, neither did the junction-stabilizing chemical, adrenomedullin, prevent transport of antibodies into the lymph node parenchyma. LEC intercellular junctions have been reported to comprise of tight junctions (Pfeiffer et al., 2008), however, SCS floor LECs were not analyzed in the study. Thus, it remains unclear what kind of junctions govern the floor of the SCS and whether it is physically possible for antibodies to flow through them.

6.7 Therapeutic applications for lymphatic transport of antibodies (III)

Therapeutic antibodies are increasingly used to treat cancer, chronic inflammatory and autoimmune diseases. Novel checkpoint inhibitors, i.e. monoclonal antibodies targeting CTLA4, PD-1 or PD-L1, have had a growing impact on the treatment of cancer. However, their delivery to solid tumors is challenging and systemic administration of these antibodies causes severe side effects, such as autoimmunity, cytokine release and vascular leakage syndromes. Hence, the development of new delivery strategies is crucial to allow efficient delivery of therapeutic antibodies into solid tumors while minimizing adverse effects. (Riley et al., 2019) By exploiting the natural draining function of the lymphatic vasculature in the skin,

s.c. administered biologically active antibodies can be directly delivered to all cells in the lymph node. Moreover, we demonstrated that antibodies exert only local functions in the draining lymph node while leaving other lymph nodes intact. S.c. administration of therapeutic antibodies would enable using lower antibody doses and restrict the effector functions of antibodies only to the draining lymph nodes. Moreover, solid tumors are known to metastasize into draining lymph nodes via the lymphatics (Alitalo and Detmar, 2012). Based on our results, low doses of tumor cell-depleting therapeutic antibodies could be delivered s.c. into the peritumoral space, where lymphatic vessels would transport them into the same draining lymph node as any metastases.

Another therapeutic approach for the s.c. delivery of antibodies is immunoactivation. We demonstrated that T cell activation could be stimulated or inhibited in the draining lymph node with the appropriate antibodies via s.c. administration. This suggests that immunoactivating antibodies could also be utilized for stimulating immune responses against foreign antigens in the draining lymph node. This would allow the development of antibody-based vaccines using therapeutic immunoactivating antibodies already available for patient care.

7 Conclusions

This study describes new lymphatic molecules that take part in regulating lymphocyte migration in the lymph node and Peyer's patches. Moreover, we describe how lymph node LECs transport lymph-borne large antigens and antibodies into the draining lymph node parenchyma, where they are available for immune cell activation or effector fuctions. The obtained results broaden the current view regarding molecules that regulate cell migration. In addition, the discovery of new functions for LECs sheds light on the filtering function of the lymph node. The findings of this study can be summarized in the following way:

1. Lymph node sinus LECs differ in the genes they express.
2. Msr1 is expressed in the lymph node SCS LECs, where it regulates migration of lymphocytes into the lymph node via the afferent lymphatics.
3. Robo4 regulates B cell turnover in Peyer's patches in addition to maintaining homeostasis in the MLN.
4. LECs transport large lymph-borne antigens and antibodies from the SCS into the draining lymph node parenchyma via fluid-phase dynamin dependent transcytosis.
5. Subcutaneously administered antibodies are delivered to the draining lymph node in a functionally intact form, which can be utilized for efficient delivery of therapeutic antibodies and local immunomodulation.

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