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Spatially targeted exocytosis of lymphatic endothelial CCL21 guides dendritic cell transmigration

Inam Liaqat

ACADEMIC DISSERTATION

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To my loved ones

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Abstract

The immune-surveillance system guards the human body from the invasion of disease-causing organisms and plays an indispensable role in the clearance of infections. To achieve that, the immune system attains a two-pronged approach. The innate immunity is critical in acute inflammation and contributes to vaccine response while adaptive immunity is needed to generate a more tailored response to infections. Carefully boosting the adaptive immune response can help to prevent tumor growth, while diminishing the immune response can protect healthy tissue, in cases of transplant rejection and autoimmune diseases. The development of new, more efficient therapies to treat inflammation associated diseases requires a deeper understanding of the processes, which control the innate and adaptive immunity.

An important aspect of the immune surveillance is the circulation of leukocytes between the blood, interstitial tissues and the lymphatic system. However, the change of compartments, requires the crossing of blood or lymphatic endothelial cell barriers, and navigation through the tissue matrix. To study how leukocytes change the compartments, the transmigration of dendritic cells (DCs) into the lymphatic vessels, provides an excellent model system. DCs constantly patrol the peripheral tissue, enter the lymphatic vessels and present the antigens to T-lymphocytes, present in the lymph nodes, which causes their activation and starts the adaptive immune response. Previous results have shown that lymphatic endothelial cells (LECs), which compose the lymphatic vessels, secrete chemokine CCL21, which attracts and facilitates the entry of DCs into the lymphatic vessels. However, it was not known how LECs guide the transmigration of DCs. Based on these the objectives of my thesis were (i) to identify the sites of DCs transmigration across the lymphatic endothelia, (ii) to identify the LEC chemokine CCL21 storage and secretory compartments, and (iii) to identify the control mechanisms of chemokine presentation in LECs.

To achieve these objectives, using extensive live microscopy we identified that, the activated DCs preferentially transmigrate through the LEC multicellular junctions, both in dermal mouse ear tissue and in primary human dermal LEC culture. Intriguingly, I found that, the chemokine CCL21 containing vesicles are targeted specifically to the LEC multicellular junctions i.e., at the site of transmigration, and are exocytosed there. To investigate the molecular mechanisms of CCL21 delivery to the LEC multicellular junctions I, first investigated the identity of the LEC secretory vesicles, which deliver the chemokine to these sites. To that end, I created a mini-library of fluorescently tagged RAB-GTPases, which are the markers of different vesicle types in cells. Using lentiviral overexpression of RAB-GTPases and fluorescently tagged CCL21, staining of endogenous CCL2, and high-resolution electron and spinning-disc microscopy, I identified the two distinct pools of chemokine CCL21 storage vesicles in LECs: a RAB3/27+ perinuclear pool within the LEC body and a RAB6+ peripheral pool at the LEC multicellular junctions. Accordingly, the RAB6+ vesicle localization of CCL21, at LEC multicellular junctions, was also confirmed in *in vivo* mouse ear pinna dermal lymphatics. Interestingly, the RAB6-interacting proteins, ELKS and RAB8, specifically localized to the LEC multicellular junctions. Inhibiting these proteins, either through siRNA

mediated knockdown or by overexpressing dominant negative mutants, ablated the exocytosis of chemokine CCL21, at the LEC multicellular junctions. This, in turn, resulted in the reduction of DC transmigration across cultured LEC monolayer.

Conclusively, my findings provide the first-ever identification of the two major chemokine secretory compartments in LECs. Additionally, my results also show how targeted exocytosis of chemokine CCL21, create the preferential sites of DC transmigration across the lymphatic endothelia. These novel findings have general significance, as they show how spatially targeted exocytosis of chemotactic cues guide the migrating leukocytes. Moreover, the targets identified in this thesis, to block chemokine secretion, can now be validated *in vivo* and later also in pre-clinical models to determine their efficiency in controlling the extent of DC transmigration into the lymphatics and the activation of the T-lymphocytes. The knowledge gained form this could, in longer term, allow the development of translational therapies aimed at controlling the extent of adaptive immune response for the treatment of inflammation associated diseases.

Tiivistelmä

Immuunijärjestelmä suojelee ihmiskehoa sairauksia aiheuttavilta organismeilta ja sillä on korvaamaton rooli infektioiden taltuttamisessa. Immuunijärjestelmässä on kaksi komponenttia, joista synnynnäinen immuniteetti tarjoaa välittömän suojan ja on tarpeellinen rokotusvasteen syntymiselle. Hankittu immuniteetti sen sijaan kohdentaa räätälöidyn puolustuksen tiettyä patogeeniä vastaan. Hankitun immuunivasteen hienovarainen tehostaminen voi estää kasvainten kasvua, kun taas hankitun immuunivasteen hillitseminen suojaa kudossiirteen hylkimisreaktiolta tai autoimmuunisairauksilta. Uusien, tehokkaampien hoitomuotojen kehittäminen tulehduksellisia sairauksia vastaan edellyttää hankittua immuniteettia säätelevien prosessien syvempää ymmärtämistä.

Tärkeä osa immuuniteettia on valkosolujen kierto verisuoniston, kudosten ja imusysteemin välillä. Tämä edellyttää valkosoluilta veri- tai imusuonen endoteelin läpäisemistä eli transmigraatiota ja navigointia kudosmatriisin läpi. Dendriittisolujen (DC) kulkeutuminen imusuoniin tarjoaa erinomaisen mallijärjestelmän valkosolujen kierron tutkimiseksi. DC:t partioivat jatkuvasti perifeerisessä kudoksessa, mistä ne siirtyvät imusuoniston kautta imusolmukkeisiin esittelemään antigeenejä, mikä puolestaan johtaa T-lymfosyyttien aktivoitumiseen ja hankitun immuunivasteen käynnistymiseen. Aiemmin julkaistut tulokset ovat osoittaneet, että imusuonen endoteelisolut (LEC), jotka muodostavat imusuonen ja erittävät CCL21-kemokiinia, ohjaavat DC:tä imusuonen läheisyyteen ja myös avustavat DC:en pääsyä imusuoneen. Ei ole kuitenkaan tiedetty, kuinka LEC:t ohjaavat DC:en transmigraatiota. Julkaistujen tutkimustulosten pohjalta väitöskirjani tavoitteina oli (i) tunnistaa DC:iden transmigraatiokohdat imusuonen endoteelissa, (ii) tunnistaa solunsisäiset CCL21-varastointi- ja eritysvesikkelityypit ja (iii) tunnistaa mekanismit, joilla kemokiinien vapautusta ja esittelyä säädellään LEC:ssä.

Väitöskirjatyössäni olen live-mikroskopiatekniikan avulla tutkinut DC:en transmigraatiota sekä hiiren korvalehden dermiksessä että ihmisen ihon primaari-LEC-viljelmissä. Molemmissa malleissa havaitsimme aktivoitujen DC:en läpäisevän imusuonen endoteelin ensisijaisesti LEC-monisoluliitosten kautta. Tutkimuksissani havaitsin myös, että CCL21-kemokiinia sisältävät vesikkelit kuljetetaan kohdennetusti LEC-monisoluliitoksiin, missä CCL21 eritetään solun ulkopuolelle. Tutkiakseni tarkemmin näiden vesikkelien identiteettiä, rakensin RAB-GTP DNA-konstrukteja, jotka tuottavat solussa fluoresoivaa proteiineja ja mahdollistavat eri vesikkelityyppien tunnistamisen. Hyödyntämällä näiden RAB-GTP-konstruktien ja fluoresoivan CCL21-kemokiinin lentiviraalista yli-ilmentämistä, endogeenisen kemokiini CCL2:n värjäystä ja korkean resoluution elektroni- ja konfokaali-mikroskopiaa, tunnistin LEC:ssä kaksi erillistä CCL21-kemokiinia varastoivaa vesikkelityyppiä: RAB3/27-positiiviset vesikkelit LEC:en keskiosissa ja RAB6-positiiviset vesikkelit LEC-monisoluliitoksissa. Vahvistin, että CCL21-kemokiini paikantuu LEC-monisoluliitoksissa oleviin RAB6-positiivisiin vesikkeleihin myös hiiren korvalehden dermiksessä *in vivo*. Myös RAB6:een sitoutuvat proteiinit, ELKS ja RAB8, paikantuivat ensisijaisesti LEC-monisoluliitoksiin. Näiden proteiinien vaimentaminen joko siRNA-välitteisesti tai mutanttiproteiinia yli-ilmentämällä, esti CCL21-kemokiinin erityksen LEC-monisoluliitoskohdissa ja vähensi DC-transmigraatiota LEC-soluviljelmissä.

Kokonaisuudessaan tutkimukseni johtivat imusuonen endoteelin CCL21 ja CCL2 kemokiinien eritysvesikkelien tunnistamiseen. Lisäksi tulokseni osoittavat, että CCL21-kemokiinin kohdennettu eritys ohjaa DC:t LEC-monisoluliitoksille ja siten edistää transmigraatiota. Yleisemmällä tasolla löydökseni osoittavat, kuinka kemokiinien paikallinen eritys ohjaa valkosolujen liikkumista. Tunnistamieni säätelyproteiinien merkittävyyttä DC:en transmigraatiossa, imusuoneen pääsyssä ja T-lymfosyyttien aktivaatiossa voidaan jatkossa tutkia *in vivo* ja myöhemmin myös prekliinisissä tautimalleissa. Väitöskirjatyöni tulokset voivat pitkällä aikavälillä mahdollistaa uusia hoitomuotoja, joilla pyritään hallitsemaan hankitun immuunivasteen voimakkuutta tulehduksellisten sairauksien hoidossa.

List of original publications

This thesis is based on the following original publication, which is referred to in the text by using Roman numeral. In addition, some unpublished data are also presented in this thesis.

I. **Liaqat, I**., Hilska, I., Saario, M., Jakobsson, E., Crivaro, M., Peranen, J., & Vaahtomeri, K. (2024). Spatially targeted chemokine exocytosis guides transmigration at lymphatic endothelial multicellular junctions. *The EMBO J,* 43(15): pp.3141-3174. DOI: 10.1038/s44318-024-00129-x

Abbreviations

ACKR1	Atypical chemokine receptor 1
BCA	Bicinchoninic acid
B-cell	B-lymphocyte
BEC	Blood endothelial cell
BMP2	Bone morphogenetic protein 2
BMP4	Bone morphogenetic protein 4
BSA	Bovine serum albumin
CCL2	Chemokine (C-C motif) ligand 2
CCL19	Chemokine (C-C motif) ligand 19
CCL21	Chemokine (C-C motif) ligand 2
CCL21ΔC	Chemokine (C-C motif) ligand 2 (with truncated C-terminal)
CCR1	C-C chemokine receptor type 1
CCR2	C-C chemokine receptor type 2
CCR3	C-C chemokine receptor type 3
CCR5	C-C chemokine receptor type 5
CCR6	C-C chemokine receptor type 6
CCR7	C-C chemokine receptor type 7
CD4	Cluster of differentiation 4
CD8α	Cluster of differentiation molecule 8-alpha
CD11b	Cluster of differentiation molecule 11B
CD11c	Cluster of differentiation molecule 11C
CD31	Cluster of differentiation 31
CD-99	Cluster of differentiation-99
cDC	Conventional dendritic cell
cDNA	Complementary deoxyribonucleic acid
cLEC	Ceiling lymphatic endothelial cell
CNS	Central nervous system
COUP-TFII	Chicken ovalbumin upstream promoter transcription factor II
CXCL1	Chemokine (C-X-C motif) ligand 1
CXCL4	Chemokine (C-X-C motif) ligand 4
CXCL12	Chemokine (C-X-C motif) ligand 12
CXCR2	C-X-C chemokine receptor 2
CXCR4	C-X-C chemokine receptor 4
DC	Dendritic cell
DC-SIGN	DC-specific ICAM-3-grabbing non-integrin molecule
DN	Dominant negative
dsRNA	Double stranded ribonucleic acid
EC1	Extracellular domain 1
EDN1	Endothelin 1
EDTA	Ethylenediamine tetra acetic acid
eNOS	Endothelial nitric oxide synthase

ELKS	RAB6-interacting protein/CAST family member 1
ESAM	Endothelial cell selective adhesion molecule
FACS	Fluorescence-activated cell sorting
FAK	Focal adhesion kinase
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
fLEC	Floor lymphatic endothelial cell
FLT4	Fms related receptor tyrosine kinase 4
FOXC2	Forkhead box C2
ECM	Extracellular matrix
GA	Gentamicin and amphotericin
GDF10	Growth differentiation factor 10
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GlyCAM-1	Glycosylation dependent cell adhesion molecule 1
GM-CSF	Granulocyte macrophage-colony stimulating factor
GPCR	G-protein coupled receptor
GTP	Guanosine-5'-triphosphate
ICAM-2	Intercellular adhesion molecule-2
ICAM-3	Intercellular adhesion molecule-3
IFN-I	Interferon type 1
JAM-A	Junctional adhesion molecule-A
JAG1	Jagged canonical notch ligand 1
L1CAM	L1 cell adhesion molecule
LAMP-1	Lysosomal associated membrane protein 1
LAMP-3	Lysosomal associated membrane protein 3
LCs	Langerhans cells
LEC	Lymphatic endothelial cell
LFA-1	Lymphocyte function-associated antigen-1
LPS	Lipopolysaccharide
LRBC	Lateral border recycling compartment
LYVE-1	Lymphatic vessel endothelial hyaluronan receptor-1
Mac-1	Macrophage-1 antigen
MHC	Major histocompatibility complex
MLC-II	Myosin light chain-II
MMP	Matrix metalloprotease
MMRN1	Multimerin 1
MMRN2	Multimerin 2
mRNA	Messenger RNA
MUNC	Mammalian uncoordinated
MVBs	Multivesicular bodies
MyRIP	Myosin VIIA and Rab interacting protein
NADPH	Nicotinamide adenine dinucleotide phosphate hydrogen
NOC2	Nucleolar complex protein 2
NR2F2	Nuclear receptor subfamily 2 group F member 2
PBS	Phosphate buffered saline
PECAM-1	Platelet endothelial cell adhesion molecule-1
pDC	Plasmacytoid dendritic cell
PDCA-1	Plasmacytoid dendritic cell marker A-1
PDGF A	platelet-derived growth factor subunit A

PDGF B	platelet-derived growth factor subunit B
Pdpn	Podoplanin
PFA	Paraformaldehyde
PIP2	Phosphatidylinositol (3,4,5)-bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
PRR	Pattern recognizing receptor
PROX1	Prospero homeobox protein 1
PSGL-1	P-selectin glycoprotein ligand-1
PTX3	Pentraxin 3
qPCR	Quantitative polymerase chain reaction
RAB-GTPase	Ras-associated binding-guanosine triphosphate
RCF	Relative centrifugal force
RIM	Regulating synaptic membrane exocytosis protein
ROCK	Rho associated kinase
SCS	Sub-capsular sinus
SEMA3A	Semaphorin 3A
SHP2	Src homology 2 domain-containing protein
siRNA	Small interfering RNA
SOX18	SRY-box transcription factor 18
STMN2	Stathmin 2
T-cell	T-lymphocyte
TGF-α	Transforming growth factor alpha
TIMPs	Tissue inhibitor of metalloproteinases
TMM	Trimmed mean of M values
TNF-α	Tumor necrosis factor-alpha
TPPP3	Tubulin Polymerization Promoting Protein Family Member 3
TRPC6	Transient receptor potential cation channel subfamily C member 6
VCAM	Vascular-cell adhesion molecule
VE-cadherin	Vascular endothelial-cadherin
VE-PTP	Vascular endothelial protein tyrosine phosphatase
VEGF-A	Vascular endothelial growth factor-A
VEGF-C	Vascular endothelial growth factor-C
VEGFR2	Vascular endothelial growth factor receptor 2
VEGFR3	Vascular endothelial growth factor receptor 3
VLA-4	Very late antigen-4/ α4β1-integrin
vLEC	Valve lymphatic endothelial cell
Wnt2	Wingless-type MMTV integration site family member 2
ZO-1	Zonula occludens-1
ZO-2	Zonula occludens-2
ZO-3	Zonula occludens-3

Introduction

The lymphatic vessel network is an essential component of the immune system. The lymphatic vessels run in parallel to the systemic circulation and supplements its function. In the body, while the blood circulatory system delivers oxygen, nutrients, and leukocytes to tissues, the lymphatic vessels facilitate the drainage of excess interstitial fluid and provides a path for its return to the blood circulation. Additionally the leukocytes which enter the lymphatic vessels pass through the lymph nodes and some of these leukocytes eventually also return to the systemic circulation (Springer, 1994, 1995; Aspelund et al., 2015). Since the arrival of antigen-presenting dendritic cells (DCs) to the lymph nodes is required for the activation of T cells, which initiate the adaptive immunity (MartIn-Fontecha et al., 2003; Forster et al., 2008; Jackson, 2019; Arasa et al., 2021), the change of compartments, i.e. the transmigration of leukocytes across the blood and the lymphatic endothelium, provides a crucial step for the functioning of the body's immune system. The breaching of the lymphatic endothelial cell barrier, by the transmigrating antigen-presenting cells, represents a bottleneck in determining the number of antigen-presenting cells in the lymph nodes and, thus, the extent of adaptive immune response. A deeper understanding of the processes which regulate leukocyte transmigration across the lymphatic endothelia is therefore essential for designing novel therapies to fight inflammation associated diseases.

Prior to my thesis, the guidance of leukocytes was better understood only at the level of transmigration across the blood endothelia. It was known that during inflammation, the release of inflammatory proteins and cytokines at the site of injury leads to an increased extravasation of leukocytes from the blood vessels into the affected tissues (Muller, 2013). Upon sensing these inflammatory cues, the leukocytes travelling in the blood flow, are attracted towards the blood vessel wall and eventually adhere (Chen & Springer, 1999). The arrested leukocytes are further guided to the site of leukocyte transmigration, where eventually the diapedesis event is orchestrated (Sumagin & Sarelius, 2010). It was also known that neutrophil extravasation across the blood endothelia occurs primarily at the multicellular junctions, where the leukocytes are actively guided, by various chemotactic cues (Sumagin & Sarelius, 2010). Several cell junction adhesion proteins and integrins are involved in this process (de Fougerolles & Springer, 1992; Xu et al., 1996; Sundd et al., 2012; Nourshargh & Alon, 2014). The entire process of leukocytes changing the compartments, is tightly regulated, ensuring

that their transmigration across the blood endothelia occurs at the right place and time. Once in the interstitial spaces of the tissue, the leukocytes navigate to the site of injury and carry out their function.

Leukocytes undergo changes in the expression of numerous proteins which facilitate their function and guided migration. Upon capturing antigens, the antigen-presenting DCs, upregulate their surface expression of CCR7 and other inflammation-associated proteins which allows the guidance of DCs towards the lymphatic vessels by sensing the secretion of chemokine CCL21 from the lymphatic endothelium (Sallusto et al., 1998; Ohl et al., 2004; Forster et al., 2012; Schineis et al., 2019). Previous studies have shown that upon secretion, the chemokine CCL21 forms a gradient in the interstitial space, which the DCs sense via their CCR7 receptor, and this causes their migration towards the lymphatics (Weber et al., 2013). Although CCR7 is upregulated as a part of the DC maturation process, some CCR7 is also expressed in steady state (Oppenheim et al., 2002; Seth et al., 2011). In homeostasis, a stochastic increase in the expression of CCR7, for example, via the disruption of inter-DC contacts, can also lead to the migration of DCs towards the lymphatics (Jiang et al., 2007; Alvarez, 2008). Once at the lymphatic vessel, the DCs breach the lymphatic endothelial cell (LEC) barrier and enter the lymphatic vessel lumen (Forster et al., 2012). Eventually, the antigen-presenting DCs migrate to the lymph node and activate the T-lymphocytes, which in turn initiates the adaptive immune response (MartIn-Fontecha et al., 2003; Forster et al., 2008; Jackson, 2019; Arasa et al., 2021).

However, the mechanisms controlling leukocyte entry into the lymphatic vessels were not fully understood. Specifically, it remained unclear if there are preferential sites of leukocytes transmigration across the lymphatic endothelia and if DCs are guided to those sites. Previous studies had shown that the DC sensing of the CCL21 via their CCR7 receptor, is also essential after their entry into the lymphatic vessels. It was shown that the CCL21 gradient also exists in the lymph nodes, which directs the DCs to the lymph node T-lymphocyte rich areas (MartIn-Fontecha et al., 2003; Ohl et al., 2004; Tal et al., 2011; Ulvmar et al., 2014). Another study showed, that the secretion of CCL21 can be triggered upon DC-LEC contact and drives the transmigration (Vaahtomeri et al., 2017). Based on this, I hypothesized that the controlled exocytosis of chemokine CCL21 determines the site of DC transmigration. The knowledge produced as a result of my project would allow the modulation of processes which regulate LEC chemokine secretion and can be used to control the extent of DC transmigration into the lymphatic vessels, across the lymphatic endothelia. To this end, in my thesis, I have characterized the secretory compartments in LECs and have identified the proteins which can be targeted to control the LEC chemokine secretion. Additionally, I have identified the existence of preferential sites of transmigration on the lymphatic endothelia, akin to transmigration of leukocytes across the blood endothelia. The knowledge gained from my project has revealed a novel cell biological

concept where targeted exocytosis of chemokine cues can be used to control the entry of transmigrating cells. Furthermore, the knowledge produced as a result of my research project could be used for pre-clinical validation of the strategies proposed in this thesis, to control DC homing to the lymph nodes, and possibly, the development of translational therapies to treat inflammation associated diseases.

Review of literature

1. General importance of cell migration

Cell migration is exclusively important in numerous biological processes. For example, during the development of an embryo, mass locomotion of cells gives rise to a gastrula (Chuai et al., 2012). This requires highly organized movement of cells, in their correct topological arrangement, for the formation of germ layers (Williams et al., 2012). Any defect in the migration of cells during this process, could lead to functional and morphological abnormalities (Sun et al., 1999). In the developing brain tissue, the neocortex contains cortical projection neurons. These multipolar, neurons migrate between the network of aligned axons and glial fibers. They constantly change their polarity until they are eventually stabilized (Tabata & Nakajima, 2003; Watanabe & Murakami, 2009; Sakakibara et al., 2014). In doing so, the single neurons travel across the central nervous system (CNS) and form spatial contacts with adjacent neurons, which eventually gives rise to functional neuronal circuits (Watanabe & Murakami, 2009; Marin et al., 2010; Cooper, 2013). Defects in the guidance of neuronal migration could lead to structural abnormalities of the brain and a variety of related disorders (Copp & Harding, 1999).

Cell migration is also crucial in an adult organism. A good example of highly orchestrated cell migration in adults is wound healing, where several types of cells migrate to the wound site and ensure tissue regeneration and closure. These include immune cells, fibroblasts, and epithelial cells, etc., each playing a significant role in different phases of healing (Clark, 2001). First, the leukocytes are attracted to the site of wound, by chemoattracting factors. The recruited monocytes, macrophages and neutrophils clear cell debris and prevent bacterial infection at the wound site. Meanwhile, the epithelial cells at the wound edges, repolarize and grow towards the wound to ensure its closure (Fenteany et al., 2000). This is then followed by the growth of new blood vessels to ensure blood flow to the newly generated skin tissue (Sorg et al., 2007).

Another example highlighting the importance of cell migration is the circulation of leukocytes throughout the body. The leukocytes are transported via the bloodstream, where they frequently exit blood vessels (a process called extravasation) and infiltrate

the surrounding tissues (Springer, 1995). While some leukocytes remain in the tissues to look for pathogens, others are circulated back into the bloodstream through the lymphatic system, ensuring efficient immune surveillance and response (Springer, 1994, 1995) (see section 2). This constant movement is crucial for maintaining the body's immune defense system.

Similarly, in metastasis, tumor cells, from a primary tumor site, can enter the systemic circulation either directly or via the lymphatic vessels. Once inside the blood vessels, the tumor cells are carried in the blood flow and are eventually arrested on the inner surface of the blood vessels, where they transmigrate out into the surrounding tissue and metastasize, giving rise to the site of secondary tumor (Muller et al., 2001; Chambers et al., 2002; Wirtz et al., 2011; Vaahtomeri & Alitalo, 2020).

Since cell migration is essential for numerous biological processes, a deeper understanding of the mechanisms that regulates the guidance of migrating cells could offer novel strategies for modulating these processes. This knowledge could lead to the development of new therapeutic interventions aimed at controlling cell movement in various contexts, such as tissue repair, immune responses, and cancer metastasis.

1.1 Directed cell migration

A review nicely postulated the 4 avid requirements of directed cellular migration. The foremost being the (1) generation of a pro-migratory signal and its (2) sensing. Next, (3) the transmission of the signal to the mediators of cell migration and finally (4) the application or execution of the migratory event (SenGupta et al., 2021). In this context, the pro-migratory signals are generated for example, by the secretion of chemokines and other inflammatory cytokines. These are sensed by specific receptors displayed on the surface of migrating cells. Additionally, cell adhesion proteins, such as integrins, also play a crucial role in the migration of leukocytes by facilitating interactions with adjacent cells and the extra cellular matrix. The interactions between the migrating leukocyte and the blood endothelia, for example, by integrins and their ligands (LFA-1/ICAM-1, VLA-4/VCAM), trigger downstream signaling events, which finally leads to the rearrangement of cell cytoskeleton and the consequential migration of cells.

Directed migration of cells therefore requires the interaction of migrating cells with their surrounding microenvironment and the sensing of various cues, which guide their movement. This dynamic process is regulated through intercellular and intracellular signaling mechanisms, which often involve the remodeling of extracellular matrix (ECM) components to facilitate movement (Schimmel et al., 2017). Cells migrate using a variety of mechanisms, including: (1) Chemotaxis, where migration is directed by chemokines secreted by nearby cells; (2) Haptotaxis, directed

migration mediated by adhesive proteins for example, integrins; (3) Durotaxis, where cells sense and respond to the stiffness of their substrate; (4) Tenertaxis, in which cells navigate through areas of least resistance; (5) Shear forces, where mechanical forces present in the local environment assist in migration (Schimmel et al., 2017); and (6) basal cell motility, a random, non-directional movement that occurs in the absence of specific guidance signals (Friedman et al., 2005; Lam and Huttenlocher, 2013; Lammermann et al., 2008).

A prime example involving all these processes is seen in the circulation of leukocytes between tissue compartments. Leukocyte extravasation from the blood vessels is a continuous process which occurs both in homeostasis and in inflammation. In homeostasis, the random movement of leukocytes enable them to patrol the interstitial tissues, in search of potential threats (Schineis et al., 2019). However, in inflammation, the migration of leukocytes is predominantly guided by chemotactic cues and various cell adhesion proteins, which are present in the tissue microenvironment (see section 2). Besides leukocytes, other cell types also display basal motility. For example, cancer cells exhibit migration which is critical for metastasis (Liu & Klominek, 2004). Similarly, during early embryonic development, migration of cells contributes to the formation of new tissues and organs (Pezeron et al., 2008). Cell migration, whether directed or random, is therefore essential not only for immune surveillance but also for processes such as development and disease progression.

1.2 Chemokine guidance

Chemokines are a subset of specialized proteins known as cytokines, which are secreted into various tissues and body compartments. Their primary role is to facilitate the migration of different cell types. Cytokines encompass a broad range of signaling molecules, including interleukins, interferons, lymphokines, and tumor necrosis factor (TNF), each playing crucial roles in producing immune responses and orchestrating cellular communication (Baggiolini, 2001; Werner & Grose, 2003). In humans about 50 different proteins are categorized as chemokines (Baggiolini, 2001; Werner & Grose, 2003). These are further sub-divided into 4 sub families namely, CC, CXC, CX_3C and XC chemokines (Murphy et al., 2000; Werner & Grose, 2003; Hughes & Nibbs, 2018), where, “C” represents the position of the consensus cysteines and “X” the number of interrupting non-conserved amino acids (Baggiolini et al., 1997; Rot & von Andrian, 2004). The chemokines function by binding to their cognate G-protein coupled receptors (GPCRs), expressed on the surface of the target cells (Murphy et al., 2000; Hughes & Nibbs, 2018). The GPCRs; (i) receive the message by binding the chemokine; (ii) decode it; and (iii) initiate the downstream cellular response (Rot & von Andrian, 2004). The secretion of chemokines and their interceptions by the cells,

carrying the receptors to detect them, stimulate the migration of the cells towards the source of chemokine secretion.

In the blood vessels, immobilization of cytokines in the vessel lumen allows the attraction and initial binding of the leukocytes to the luminal surface of the blood vessels (Rot, 1992; Tanaka et al., 1993). Where, the chemokines attract the leukocytes, other factors like cell surface integrins provide adhesion and activate the migratory machinery of the attaching leukocyte (Gorina et al., 2014) (see section 2). Similarly, the DCs, patrolling in the interstitial spaces, recognize the presence of chemokine CCL21, via their CCR7 receptor, and are attracted towards the source of chemokine secretion (Forster et al., 1999; Ohl et al., 2004). The chemokine CCL21 is secreted by the LECs, which form the surface of the lymphatic vessels, or by the fibroblast reticular cells present in the sub-capsular sinus and parenchyma of the lymph node (Luther et al., 2000; Johnson & Jackson, 2010; Vaahtomeri et al., 2017).

Once secreted into the surrounding tissue, the chemokines can bind to the extracellular matrix (ECM) or to the cell surfaces, via proteoglycans, as shown for chemokine CCL21 and CXCL4 (PF-4) (Petersen et al., 1998; Bao et al., 2010; Weber et al., 2013). Anchoring of chemokines allows the formation of interstitial chemokine gradients (Weber et al., 2013). However, in the lymph node, the CCL21 gradient is formed via its binding to sink receptor ACKR4 (CCRL1), expressed by a subset of LECs, present in the sub-capsular sinus (Ulvmar et al., 2014).

Some chemokines, however, have a lower affinity for the extracellular matrix (ECM) and other binding components, preventing them from anchoring, as reported for chemokine CCL19 or cleaved chemokine CCL21 (without its C-terminus), which remains freely dispersed within the surrounding tissue environment, forming soluble gradients (Schumann et al., 2010). Such gradients, are generally formed by the flow of interstitial fluid in the tissue matrix or for example the flow of lymph in the lymphatic vessel lumen (Schumann et al., 2010; Russo et al., 2016). Other reports have shown that limited diffusion is required for the formation of fibroblast growth factor gradient (Makarenkova et al., 2009; Yu et al., 2009) and chemokine gradient (Weber et al., 2013). In the absence of limited diffusion (purely soluble), the chemokine CCL21 gradient tends to flatten. It is, however, the anchored chemokine gradient formed by the immobilized chemokine, which, after the initial attraction, further leads the migrating antigen-presenting DCs to the lymphatics (Weber et al., 2013; Schwarz et al., 2017). The chemokine is therefore necessary for the initial attraction of the DCs towards the lymphatics. However, what guides their migration into the lymphatic vessel lumen, across the LEC barrier, was not known and has been explored in this thesis.

1.3 Principle of RAB-GTPase based vesicular targeting

Chemokine and other secretory proteins are packaged in membrane-bound vesicles, after their production (Lacy, 2015; Jiao et al., 2024). These vesicles are eventually trafficked to the plasma membrane for secretion. After their arrival, the vesicles must dock and fuse to the plasma membrane and ultimately release their contents into to the extracellular space. This event is termed as exocytosis. The trafficking of the secretory vesicles is regulated by a specialized set of proteins called the RAB-GTPases (Fukuda, 2008; Wandinger-Ness & Zerial, 2014; Zhen & Stenmark, 2015). The RAB-GTPases orchestrate the motility, docking and fusion of vesicles with the targeted membranes (Pfeffer, 2001; Segev, 2001; Zerial & McBride, 2001).

To perform their function the RABs must first be activated. To do that, the inactive guanosine diphosphate (GDP)-bound RAB-GTPase must convert to guanosine triphosphate (GTP)-bound conformation (Fukuda, 2008). This conversion is controlled by guanine nucleotide exchange factors (GEFs), which facilitate GDP-to-GTP exchange to activate RAB-GTPases, and the GTPase-activating proteins (GAPs), which accelerate the hydrolysis of GTP to inhibit RAB-GTPase function (Eathiraj et al., 2005; Lee et al., 2009). Activated RAB-GTPases are geranylgeranylated at their C-terminus, which enables stable binding with the lipid bilayers (Leung et al., 2007; Shinde & Maddika, 2018). The RAB-GTPases are ultimately recycled via the action of RAB GDP dissociation inhibitor (GDI), which allows their removal and, subsequent, recruitment to the appropriate membrane compartments (Soldati et al., 1994; Goody et al., 2005). Many cellular processes use membrane targeted trafficking of vesicles and therefore the RAB-GTPases can control them, for example cell migration, homeostasis, secretion of paracrine effectors (van Ijzendoorn et al., 2003; Militello & Colombo, 2013; Numrich & Ungermann, 2014; De Franceschi et al., 2015).

Numerous RAB GTPases have been characterized in humans (Homma et al., 2020). The RAB-GTPases commonly associated with secretory vesicles include, RAB1A, RAB2A, RAB3A/B/C/D, RAB6A, RAB7A, RAB26, RAB27 and RAB37 (Wandinger-Ness & Zerial, 2014; Zhen & Stenmark, 2015). From this list the RAB6 and RAB7 are associated with Golgi-derived vesicles and late endosomes, respectively (Soldati et al., 1995; White et al., 1999; Bucci et al., 2000; Wandinger-Ness & Zerial, 2014; Miserey-Lenkei et al., 2017). Despite their uniqueness many RAB-GTPases might localize to the same type of vesicles, for example RAB3A/B/C/D, RAB27A/B, and RAB37 have been previously shown to be associated with the traditional dense-core secretory vesicles (Pereira-Leal & Seabra, 2001; Tsuboi & Fukuda, 2006). The examples of dense-core granules include, the Weible-Palade bodies in BECs, which are the RAB27 positive dense-core secretory granules (McCormack et al., 2017; Nass et al., 2021); RAB3A/B/C, RAB26, and RAB27B positive synaptic vesicles (Takamori et al., 2006); RAB3A, RAB26, and RAB27A positive pancreatic acinar zymogen granules (Rindler et al., 2007); RAB27A and RAB37 positive NK cell dense-core secretory lysosomes (Casey

et al., 2007); and the RAB3A/C/D and RAB37 positive insulin secretory dense-core granules (Brunner et al., 2007). A previous study reported that downregulating RAB3A and RAB27A impaired the secretion of insulin in type 2 diabetes (Abderrahmani et al., 2006).

RAB-GTPases also require specific effector proteins to support their function. Effector proteins have high specificity towards activated RAB-GTPases (Grosshans et al., 2006; Khan, 2013). These proteins are essential for, for example, the transport and docking of different vesicles to the site of exocytosis. Several studies have reported the effectors of RAB3 and RAB27 in numerous cell types. These effector proteins include, for example synaptotagmin-like protein-4a (Slp4a) in the RAB27A+ insulin granules, which is required for the exocytosis of insulin in pancreatic β-cells (Torii et al., 2002; Yi et al., 2002); Slp homologue lacking C2 domains (Slac2-a-c) helps in the tethering of RAB27A+ melanosomes by fostering interaction with myosin VIIa in retinal epithelial cells (El-Amraoui et al., 2002); Slac2a mediates interaction between myosin Va and RAB27A. Blocking this interaction causes Griscelli syndrome (GS) (Menasche et al., 2000; Seabra et al., 2002); synaptotagmin-like protein-2a (Slp2-a) is needed for the binding of RAB27A+ melanosomes and glucagon granules to the plasma membrane (Kuroda & Fukuda, 2004; Yu et al., 2007); and Munc13-4 which is essential for the tethering of secretory lysosomes to the plasma membrane in cytotoxic T lymphocytes (Elstak et al., 2011).

A previous study has reported that also RAB6 interact with RAB8 and MICAL3 to regulate the docking and fusion of exocytic vesicles in HeLa cells (Grigoriev et al., 2011). Furthermore, it was shown that traditional Golgi-derived RAB6+ vesicles associate with RAB6 interacting protein 1 (Rab6IP1), which facilitates their transport from the Golgi apparatus to the plasma membrane (Jasmin et al., 1992; Martinez et al., 1994; Martinez et al., 1997; Girod et al., 1999; White et al., 1999; Opdam et al., 2000; Young et al., 2005; Del Nery et al., 2006; Utskarpen et al., 2006; Recacha et al., 2009). Conclusively, RAB-GTPases and their partners play a crucial role in the regulation of vesicular trafficking in the cells and therefore control numerous cellular processes. Due to their vesicle-type specificity, the RAB-GTPases can be used to target different membrane bound compartments in cells and present excellent tools to manipulate and study secretory routes in cells.

2. Circulation of leukocytes between the blood and lymphatic systems

The two main circulatory systems in the body are the cardiovascular and the lymphatic systems. Both of these systems are essential in the circulation of leukocytes and other biomolecules around the body. The blood vessels are responsible for transporting oxygen, carbon dioxide, nutrients, leukocytes and for removing cellular waste. Conversely, the lymphatic vessel network runs in parallel to the blood vessels, and is needed for the drainage of tissue fluid, transport and maturation of leukocytes and the absorption of gastrointestinal lipids (Aspelund et al., 2016; Jackson, 2019). The blood vessels comprise of arteries, veins and capillaries, and are connected in a circuit. This allows circular flow of blood and nutrients in the blood vessels. In contrast, the lymphatic vessels consist of blind-ended capillaries, which merge into the larger lymphatic vessels. The fluid in the lymphatic vessels, flows from the interstitial space into the lumen of the lymphatic vessels, passing through lymph nodes, it is drained into the venous system and is ultimately, returned to the heart (Randolph et al., 2005; Aspelund et al., 2016). The interconnection between the blood and the lymphatic vessels, therefore, provides a pathway for the circulation of leukocytes, which is very essential in homeostasis and in inflammation (as explained in the sections below).

2.1 Blood and lymphatic vessel architecture

2.1.1 Architecture of the blood vessels

During early development, the first organ system formed in the body is the cardiovascular system (Olson & Srivastava, 1996). This comprises of the heart and the blood vessels (arteries and veins). During gastrulation, the progenitor cells of the hemangioblast differentiate into endothelial cells under the control of vascular endothelial growth factor receptor (VEGFR2/VEGF-a) mediated signaling (Eichmann et al., 1997). During development, the initial differentiated cells attain a tubular conformation, forming the first conduits, in which the blood is transported (Rossant & Howard, 2002). These differentiated endothelial cells further give rise to the primary capillary plexus which is ultimately remodeled into arteries and veins (Eichmann et al., 2005). A fully developed blood vascular system comprises of the main arteries which subdivide into smaller arteries, arterioles and finally the capillaries, reaching all organs and tissues in the body. These capillaries further merge into slightly larger venules, which merge into bigger veins and are finally connected back to the heart. Unlike the arteries, the veins also have valves, which stops the back flow of blood. This forms the closed circuit for the circulation of blood in humans (Udan et al., 2013). The surface of the blood vessels is lined with a monolayer of blood

endothelial cells (BEC), which are connected to the adjacent BECs through lateral cell junctions and are sealed by cell adhesion proteins (Muller et al., 1993). This presents a barrier to prevent leakage of blood components into the surrounding tissue and limits the entry of tissue fluid and cells into the blood stream.

Additionally, the blood endothelial cell surface is further decorated with numerous proteins, including cell surface receptors, adhesion molecules, proteoglycans etc., which serve different functions related to cell-cell contact, vascular permeability and sensing of mechanical and chemical cues. On the luminal side of the blood vessel, the endothelial cell surface is covered with glycocalyx which is a sheet of proteoglycans (Singh et al., 2007). While, on the abluminal side, the endothelial cells in turn interact with basement membrane, pericytes and extracellular matrix (Rossant & Howard, 2002). In the larger blood vessels and veins, the abluminal layer of endothelial cells is further reinforced by an extensive matrix, along with an additional layer of smooth muscle cells, elastic fibers, and connective tissue. This provides elasticity to the blood vessels, to sustain the high pressure of blood. However, most of the exchange of solutes and leukocytes occur in the capillaries and post-capillary venules. Depending on their function, these smaller vessels are devoid of reinforcements completely, making the transport of material easier (Udan et al., 2013).

2.1.2 Architecture of the lymphatic vessels

Parallel to the blood circulatory system, the lymphatic network extends throughout most organs and peripheral tissues. Like the blood vessels, the surface of lymphatic vessel is formed by a monolayer of LECs. It was previously believed that the first lymphatic vessels originate from the lateral plate mesoderm. To do that, the BECs, present in the posterior cardinal vein, differentiate into LECs around embryonic stage E9.75. This process was thought to be regulated by transcription factors SOX18 and NR2F2 (COUP-TFII), which induces the expression of PROX1. This shift triggers the downregulation of blood-specific genes and the upregulation of lymphatic-specific markers, such as podoplanin (Pdpn), Foxc2, and Flt4 (VEGFR3), leading to the acquisition of lymphatic characteristics (Kaipainen et al., 1995; Petrova et al., 2002; Wigle et al., 2002; Petrova et al., 2004; Francois et al., 2008; Bertozzi et al., 2010; Srinivasan et al., 2010). The increased expression of VEGFR3 makes these cells responsive to VEGF-C, secreted by surrounding mesenchymal tissue, enabling them to separate from the cardinal vein and form the initial lymph sacs. From these lymph sacs, the complex lymphatic system develops further through sprouting, a process known as lymphangiogenesis (Karkkainen et al., 2004; Yamazaki et al., 2009; Udan et al., 2013).

However, recent studies have revealed that while cervical and thoracic lymphatic vessels arise from venous-derived lymph sacs via sprouting, the lymphatic vessels in

the lumbar region and dorsal midline skin are formed from non-venous-derived cells that differentiate into LECs, a process called lymph vasculogenesis (Martinez-Corral et al., 2015). These mesenteric lymphatic vessels originate from the cKit lineage cells, which have a hemogenic endothelial origin (Stanczuk et al., 2015). This new understanding challenges the traditional view that lymphatic vessels solely originate from the lateral plate mesoderm.

Moreover, emerging evidence has recently shown that some of the earliest PROX1+ progenitor cells are actually derived from the paraxial mesoderm, not the lateral plate mesoderm. It was further suggested that muscle progenitor cells and multipotent progenitors can also give rise to LECs, highlighting a more complex and diverse origin for LECs (Stone & Stainier, 2019).

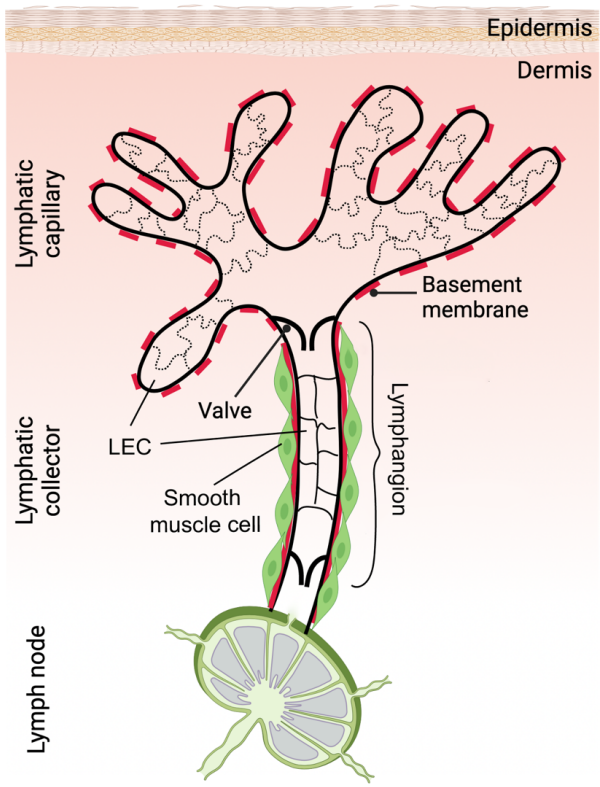


Figure 1: Lymphatic vessel architecture. The schematic shows a simplified diagram of blind-ended lymphatic capillaries, collector and lymph node. These capillaries are surrounded by a discontinuous basement membrane (red) and exhibit discontinuous cell junctions in homeostasis. The initial lymphatic capillaries merge into collecting vessels, which are surrounded by a continuous basement membrane (red) and smooth muscle cells. This gives rise to individual units of the collecting vessel called “lymphangion”. The smooth muscle cells contract and expand to provide pumping force required to move the lymph through the collecting vessel, to the draining lymph node. The lymphatic endothelial cell

junctions in the collecting vessels are continuous and the cells exhibit elongated morphology unlike the irregular or “oak-leaf” like shape in the lymphatic capillaries. The collecting vessels have valves which ensure unidirectional flow of the lymph towards the draining lymph node. This schematic is modified from (Jackson et al., 2019). The schematic was created using BioRender.com.

Unlike the blood circulatory system, the lymphatic system is not connected in a circuit. The lymphatic vessels consist of the blind-ended lymphatic capillaries, which are also called the initial lymphatics. The initial lymphatic capillaries converge into pre-collectors and collectors, followed by a network of lymph nodes connected in a series, and then the larger lymphatic vessels, which drain into the venous system (Schineis et al., 2019; Zhang et al., 2020). The lymphatic capillaries cover the interstitial space between the tissues, while the comparatively bigger collecting vessels exist in close proximity of the blood vessels. The lymphatic capillaries are characterized by the presence of highly perforated basement membrane surrounding their surface. However, the collecting vessels are surrounded by a continuous basement membrane and smooth muscle cells (Pflicke & Sixt, 2009) (Figure 1). Additionally, the collecting vessels display the presence of valves, which prevent the back-flow of the lymph (Tammela & Alitalo, 2010) (Figure 1).

Moreover, the LECs in the collecting vessels display elongated morphology as compared to the “oak leaf-like” shape of the LECs in the lymphatic capillaries (Baluk et al., 2007) (Figure 1). The collecting lymphatic vessels are made up of functional units called "lymphangion". These units, through the coordinated contraction and relaxation of surrounding smooth muscle cells, create a pumping motion in the vessel, facilitating the active movement of lymphatic fluid (lymph) towards the draining lymph node (Tammela & Alitalo, 2010; Schulte-Merker et al., 2011).

The lymphatic capillaries express LYVE1, which is a receptor for hyaluronan. This is not expressed in the collectors (Makinen et al., 2005). Additionally, the lymphatic capillaries also have a comparatively higher expression of chemokine CCL21, which via binding to its cognate receptor CCR7, present on the surface of the antigen-presenting DCs, allows the attraction and migration of DCs preferentially through the lymphatic capillaries (Ulvmar & Makinen, 2016; Arasa et al., 2021). Recent RNA-sequencing (RNA-seq) studies have been instrumental in differentiating between the different sub-types of LECs based on their gene expression data (see section 2.3).

2.2 Function of lymphatic vessels

During the circulation of blood in the systemic circulation, some of the plasma and leukocytes exits the blood vessels and enter the interstitial spaces of the surrounding tissues. Some of these leukocytes and the tissue fluid re-enters the blood circulation

via the lymphatic capillaries (Dandie et al., 1994; Randolph et al., 2005; Hampton et al., 2015; Hunter et al., 2016). The total volume of the tissue fluid in the interstitial spaces is returned to the systemic circulation through the lymphatic system every nine hours (Levick & Michel, 2010).

After entering the lymphatic vessel lumen, the lymph moves via the capillaries to the pre-collectors and collector vessels. The collectors further empty into a series of lymph nodes (secondary lymphoid organ), where immune cell activation and maturation occurs (Aspelund et al., 2016; Jackson, 2019). Moving through, the lymphatic fluid is ultimately drained into the right lymphatic trunk and the thoracic duct, which merges into the systemic circulation by joining at the junction of internal jugular vein and subclavian vein (Randolph et al., 2005; Aspelund et al., 2016).

In the intestines, the initial lymphatics are responsible for absorbing dietary fats as chylomicrons (Randolph & Miller, 2014). The mesenteric lymphatics drain lymph back to the blood flow via the thoracic duct (Petrova & Koh, 2020). Based on the direction of lymph, the lymphatic vessel network can be divided into “afferent” lymphatics i.e., which carry unfiltered lymph from tissues to the lymph node, and the “efferent” lymphatics, which carry the filtered lymph from the first lymph node to the next subsequent lymph node or back to the blood circulation (Haig et al., 1999).

Therefore, the blood and the lymphatic vasculature provide a means by which leukocytes and other substances are circulated continuously in the body. This dynamic exchange of material is crucial in the normal functioning of bodily processes including immunity.

2.3 Characterization of LEC sub-types

Modern RNA sequencing techniques have opened new avenues of research for addressing complex biological questions like the lineage classification of cells and their sub-types. Bulk RNA-sequencing (Bulk RNA-seq) data can provide gene expression patterns representing the average read-out of all sequenced cells. On the other hand, the extensive resolution of single-cell RNA sequencing (scRNA-seq) can be used to characterize the sub-populations of cells and to understand the key transcriptional programs and signaling pathways. Computational analysis of the obtained sequencing data is then required to generate a comprehensible readout.

Using the extensive power of RNA-seq techniques, novel information about the development and functional states of LECs and DCs has been elucidated in several studies (Villani et al., 2017; Takeda et al., 2019; Xiang et al., 2020; Chen et al., 2021; Gonzalez-Loyola et al., 2021; Sibler et al., 2021; Petkova et al., 2023; Protti &

Spreafico, 2024). This information has been vital in ascribing mRNA signatures to the identified LEC and DC sub-types, including signatures related to immune regulation, antigen presentation, and tissue homeostasis. Moreover, scRNA-seq has also been used to understand how different cellular sub-populations interact (Xiang et al., 2020). For example, scRNA-seq and bulk RNA-seq data from DCs has been combined to understand the heterogeneity and functional role of DCs in squamous cell carcinoma and clear cell renal cell carcinoma models (Shi et al., 2024; Ye et al., 2024). Despite its immense power, the interpretation of RNA-seq data must be done carefully, as it is possible that some genes are not detected, due to the technical limitations of the technique or the analysis model used, instead of their actual absence.

Nonetheless, the power of scRNA seq approach has allowed the identification of LEC sub-types. In mesenteries, following LEC sub-types have been reported: capillary LECs (capLEC), pre-collector LECs (pre.collLEC), collector LECs (collLEC), valve LECs (vLEC), and the newly characterized subtypes; interferon producing LECs (IfnLEC), and proliferative LECs (prolifLEC) (Alejandra González-Loyola, 2021). Intriguingly, it was also reported that the capLECs, which form the blind-ended capillary network of the initial lymphatics, express lower level of *Piezo2*– mechanosensitive ion channel protein as compared to collLECs and vLECs (Gonzalez-Loyola, 2021). This is obvious since the blind-ended capillaries experience a lower magnitude of transmural flow. On the other hand, capLECs express higher level of genes which regulate microtubule dynamics including, Stathmin-2 (*Stmn2*) and Tubulin polymerization promoting protein family member 3 (*Tppp3*). Additionally, they also show higher expression of decoy receptor; Atypical chemokine receptor 2 (*Ackr2*), which shows how initial lymphatics control chemokine signaling. On the contrary, the collLECs also exhibit a higher expression of Bone morphogenic protein 4 (*Bmp4*), Platelet-derived growth factors (*Pdgfa* and *Pdgfb*), Transforming growth factor alpha (*TGF-α*), and Wingless-type MMTV integration site family member 2 (*Wnt2*), *which* are related to pericyte and fibroblast recruitment and proliferation. Furthermore, many other genes, which had never been reported in collLECs, were also identified, which point to their newly anticipated functions (Gonzalez-Loyola et al., 2021).

Apart for this, another study has reported scRNA-seq based categorization of mouse dermal LECs sub-populations which includes; capillary LECs (Cap), collector LECs (Col), valve LECs (Valve), proliferative capillary LECs (Cap prolif), and Pentraxin-3 (Ptx3)+ capillary LECs (Ptx3 Cap) (also called iLECs) (Milena Petkova, 2023). The identified LEC sub-types were similar to mesenteric LECs except iLECs. Intriguingly, it was shown that the iLEC population express chemokine CCL2 and other pro-inflammatory signature genes which control the recruitment of pro-lymphangiogenic macrophages. This provided new knowledge about how peripheral lymphatic vessels also contribute to organizing inflammatory events locally.

The scRNA seq data of peripheral LECs reported in the above mentioned studies (Alejandra González-Loyola, 2021; Milena Petkova, 2023), and bulk RNA-seq data from LECs (unpublished results, this thesis) have been compared in this thesis to visualize the expression levels of different RAB-GTPases and chemokines (see figures 10 and 11 in the results sections 8.2 and 8.3 of this thesis).

Like the lymphatic capillaries and the collecting vessels, the LNs are also composed of LECs, which line the subcapsular (SCS) and medullary sinuses. Apart from LECs, lymphocytes and other stromal cells including the fibroblast reticular cells (FBR) are also present in the LNs (Takeda et al., 2019). Antigen-presenting DCs, which enter the LN through the afferent lymphatic vessel, must first transmigrate across the LEC barrier in the sub-capsular sinus (SCS), to reach the T-cell rich zones in the LN (MartIn-Fontecha et al., 2003; Forster et al., 2008; Jackson, 2019). Recent RNA-seq studies have shown that the LECs present in the LNs are different from the peripheral LECs, owing to their distinct expression patterns (Takeda et al., 2019; Xiang et al., 2020). Based on these studies, the LN LECs were segregated into four major sub-sets, including the (1) ceiling LECs (cLECs) and, (2) floor LECs (fLECs) of the sub-capsular sinus (SCS), (3) Ptx3-LECs of the paracortical and medullary sinus, and (4) Macrophage Receptor with Collagenous Structure (MARCO)-LECs of the perifollicular medullary sinus (Xiang et al., 2020). These LECs sub-sets are common to human and mouse LNs. However, a newly described LEC subset, unique to human LNs, the *MFAP4*+ LECs, was also identified in the medulla capsule of the LN (Takeda et al., 2019).

A previous study had reported that the cLECs express atypical chemokine receptor (ACKR4), which allows the creation of the gradient of CCL21 in the SCS of the LN. This ultimately allows the attraction of DCs towards the fLECs and their subsequent transmigration into the LN paracortex (Ulvmar et al., 2014). Recent scRNA-seq data has revealed that besides that, the cLECs also express multimerins (*Mmrn1 and Mmrn2*), *Pdgfa, Pdgfb*, Endothelin 1 (*Edn1*), and Jagged-1 (*Jag1*), which allows the interaction of cLECs with the connective tissue of the SCS (Xiang et al., 2020). Additionally, the cLECs also express *Bmp4* and Growth Differentiation Factor 10 (*Gdf10*), which distinguishes them from the fLECS (Xiang et al., 2020). On the other hand, the fLECs, which present the transmigration barrier to the lymph borne DCs in the SCS, express chemoattractants CCL20, CXCL1 and cell-adhesion proteins ICAM-1, VCAM-1, and GlyCAM-1 (Xiang et al., 2020). These proteins facilitate the transmigration of DCs across the fLECs, into the LN parenchyma. In addition to that, the fLECs are different from cLECs due to their expression of *Bmp2,* and in mice the expression of mucosal vascular addressin cell adhesion molecule 1 (*MAdCAM-1*) (Xiang et al., 2020).

The Ptx3-LECs, on the hand show higher expression of genes associated with lymphangiogenesis and the egress of lymphocytes, which is needed for the remodeling of the lymph node and the recruitment of immune cells to the LNs (Xiang et al., 2020). The MARCO-LECs in turn express genes related to the recruitment of macrophages, lymphangiogenesis, immune response, protein-protein interaction and enzymatic cleavage (immune complement and coagulation cascades) (Martens et al., 2006; Takeda et al., 2019; Fujimoto et al., 2020; Xiang et al., 2020). Another recent study has shown that MARCO-LECs are able to capture arthritogenic alphavirus in the draining LN to limit its dissemination (Carpentier et al., 2021). Conclusively, RNA-seq based techniques have allowed characterization of cellular sub-types and in understanding their function. However, despite the similarities between the human and mouse LN LECs, certain dissimilarities also exist. The key markers differentiating mouse and human LN LECs are discussed in an excellent review article (Takeda et al., 2019).

2.4 Types of leukocytes

Various types of leukocytes are circulated in the body, via the blood and the lymphatic vasculature. The leukocytes are exclusively essential as the primary defenders in the body's immune system. They are broadly classified into two main groups: granulocytes (including basophils, eosinophils, and neutrophils) and agranulocytes (comprising DCs, T-lymphocytes, B-lymphocytes, and monocytes). Leukocytes originate from either myeloid or lymphoid common progenitors which primarily develop in the bone marrow (Monga et al., 2022). Additionally, blood cells such as megakaryocytes and erythrocytes also originate from the common myeloid progenitor (Orkin & Zon, 2008; Monga et al., 2022). Lineage-tracing studies have demonstrated that these progenitors can be traced back to the hematopoietic stem cells. Upon differentiation, progenitor cells give rise to various specialized immune cells. Notably, some monocytes can also further differentiate into DCs (Marzaioli et al., 2020) or macrophages (Mass et al., 2023), giving birth to the tissue resident leukocytes. Despite the somewhat controversial view about the origin of DC sub-types, it is generally thought that the conventional DCs originate from the myeloid precursor while, the plasmacytoid DCs, originate from the lymphoid progenitors (Figure 2).

Other than their progenitor origin, the leukocytes can also be further categorized into various sub-subtypes based on their functions and specific membrane markers. However, for the sake of simplicity, here only a simplified classification of common leukocyte subtypes, based on their origin, is presented (Figure 2).

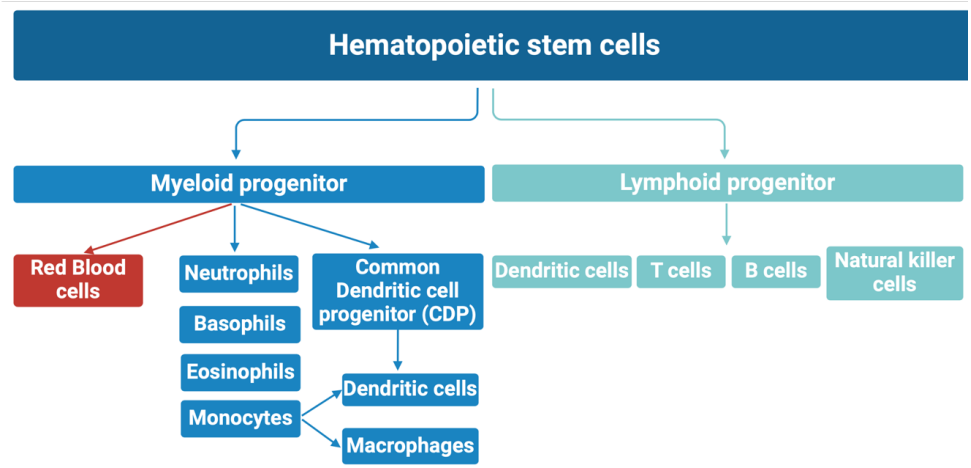


Figure 2: Types of leukocytes. Flow-chart shows common sub-types of leukocytes. The hematopoietic stem cells in the bone marrow differentiate into myeloid and lymphoid progenitors. The myeloid progenitor can further differentiate into red blood cells (erythrocytes), neutrophils, basophils, eosinophils, monocytes and common dendritic cell progenitor (CDP). Monocytes can further differentiate into dendritic cells and macrophages, especially in inflammation (Guilliams et al., 2014). However, in homeostasis most of the dendritic cells originate from the myeloid progenitor derived CDP (Sichien et al., 2017) (see section 3.2). On the other hand, the lymphoid progenitor differentiates into dendritic cells (Manz et al., 2001), T-cells, B-cells, and natural killer cells. This figure was created using BioRender.com.

Leukocytes may undergo additional maturation and activation in various tissues. For example, T-cells originate in the bone marrow, mature in the thymus, and are subsequently activated in the lymph nodes (Spits, 2002; Bousso, 2008). Conversely, other leukocytes, such as DCs, mature in the bone marrow and are activated in the peripheral tissues (Sallusto et al., 1998; Liu & Nussenzweig, 2010; Sichien et al., 2017).

2.5 Leukocyte circulation

After their production, the leukocytes are transported across the body, via the blood flow. At some point during their lifetime, the leukocytes extravasate the blood vessels and enter the surrounding tissues. Some of the leukocyte sub-types return to the systemic circulation while, others remain resident in the tissues (see Table 1). One of the ways by which leukocytes from the tissues return to the blood flow, is via the lymphatics. This route is possible because the cardiovascular and the lymphatic systems are connected to each other (see Figure 3), and exchange of immune cells takes place between them.

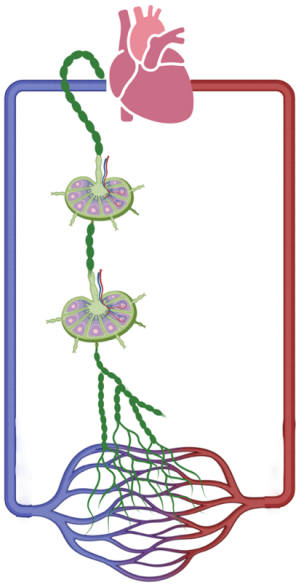


Figure 3: leukocyte circulation. Schematic shows that the blood (arteries: red, veins: purple) and lymphatic vasculature (green) are connected. The initial lymphatics surround the blood capillaries, where tissue fluid and most of the leukocytes enter the lymphatics. They are then transported via larger lymphatic collectors to the lymph nodes, which are connected in a series. Finally, the lymphatic vessels drain into the blood vessels thereby providing a route for the return of leukocytes and tissue fluid to the systemic circulation. This schematic is modified from (Aspelund et al., 2016). The schematic was created using BioRender.com.

Leukocyte circulation is essential for the continuous immune surveillance function of the body. To support that, various immune cells constantly re-enter the blood flow. Pearson et al., 1976, reported that radioactively labelled fetal sheep T-lymphocytes, infused intravenously, returned to the thoracic duct after 10-18h (Pearson et al., 1976). This was also shown by Cahill et al., 1977, in adult sheep, where they reported a similar time frame for the return of radioactively labelled T-lymphocytes, back to the blood flow (Cahill et al., 1977). A more recent study showed that T-cells enter the lymphatics, from the blood via the high endothelial venules (HEV) in the lymph node. Alternatively, T-cells can also be carried from the peripheral tissue to the lymph node via the afferent lymphatics. From the lymph node, they then leave via the efferent lymphatics (Randolph et al., 2005; Hunter et al., 2016).

However, the route taken for the recirculation depends on the presence of pathogenic encounter. For example, neutrophil return, from the site of a sterile wound, occurs mostly through the blood vessels but upon bacterial infection, most of the neutrophils enter the lymphatic vessels (Hampton et al., 2015). Some neutrophils which enter the lymphatics are returned to the systemic flow and subsequently back to the bone

marrow (Wang et al., 2017). However, most of the neutrophils die at the site of inflammation, owing to apoptosis and their short life-span (Savill et al., 1989; Scheiermann et al., 2015). Table 1 summarizes the fate of leukocyte sub-types in entering the lymph nodes and in re-entering the systemic circulation.

Table 1: Fate of leukocytes: The table enlists leukocyte subtypes and shows if they enter the lymph nodes or are recirculated by re-entering the blood circulation.

Type of leukocyte	Entry into the lymph nodes	Recirculation via blood vessels
Basophils	Yes; upon inflammation (Kim et al., 2010; Miyake & Karasuyama, 2017)	No; short life-span (Ohnmacht & Voehringer, 2009)
Eosinophils	Yes; upon inflammation (Shi et al., 2000; Duez et al., 2004)	No (Shi, 2004; Ramirez et al., 2018; Day et al., 2024)
Neutrophils	Yes; upon bacterial infection/ inflammation (Abadie et al., 2005; Maletto et al., 2006; Chtanova et al., 2008; Yang et al., 2010; Beauvillain et al., 2011; Hampton et al., 2015)	Limited; mostly die in the tissues (Mathias et al., 2006; Woodfin et al., 2011; Scheiermann et al., 2015; Wang et al., 2017)
Monocytes	Yes; upon inflammation (Janatpour et al., 2001; Muller, 2001; Palframan et al., 2001)	No; they differentiate into subtypes in tissues and lymph node (Randolph et al., 1999; Patel et al., 2017)
Macrophages	Yes; lymph node resident sub-population of macrophages (Muller, 2001; Louie & Liao, 2019)	No; macrophages are killed at the site of injury. However, tissue and lymph node resident sub-populations of macrophages also exist (Kuhlmann et al., 2001; Chtanova et al., 2008; Cox et al., 2021)
Dendritic cells	Yes (Forster et al., 2008; Forster et al., 2012; Russo et al., 2013)	Limited; subtype specific. Only 10% migratory DCs return to circulation (Dandie et al., 1994). Most migratory DCs die in the lymph nodes (Tomura et al., 2014; Austyn, 2016)
T cells	Yes (Marchesi & Gowans, 1964; von Andrian & Mempel, 2003; Forster et al., 2012)	Yes; subtype specific (Gowans & Knight, 1964; Ford, 1968, 1969; Cyster, 2005; Lo et al., 2005)
B cells	Yes (Marchesi & Gowans, 1964; von Andrian & Mempel, 2003)	Yes; subtype specific (Gowans & Knight, 1964; Ford, 1968, 1969; Cyster, 2005; Lo et al., 2005)
Natural killer cells	Yes (Ferlazzo et al., 2004; Freud et al., 2005; Cruz de Casas et al., 2024)	Yes; subtype specific (Carrega & Ferlazzo, 2012; Carrega et al., 2014; Hamilton et al., 2017)

Unlike the neutrophils, the antigen presenting DCs exclusively use the lymphatic route in the lymph node for the return of a small population of migrating DCs back to the blood flow. Unlike the neutrophils, most of the DCs, after entering the lymphatics never leave the lymph node (Tomura et al., 2014; Austyn, 2016). Only about 10% of DCs, which enter the lymphatics, are returned back to the systemic flow (Dandie et al., 1994). Their entry into the lymphatic vessels is necessary for the presentation of antigens to the T-cells in the lymph nodes. This is crucial for peripheral immune tolerance and for the activation of adaptive immunity (Forster et al., 2012; Aspelund et al., 2016; Audiger et al., 2017).

However, some leukocyte sub-types mostly do not return from the tissues back to the blood stream, for example the granulocytes (Springer, 1995). Amongst these, some leukocytes develop further into tissue resident population of specialized immune cells, for example the macrophages (Ginhoux & Guilliams, 2016) and the central memory and effector memory T-cells (Masopust & Soerens, 2019) (Sallusto et al., 2004) (Table 1). These cells provide continuous protection and represent the first responders to fight against incurred infections.

3. Innate and adaptive immunity

The body's immune system comprises of a variety of complex processes, which function together to dynamically adjust the required immune response, according to the need. The continuous surveillance provided by the circulating leukocytes provides immediate immune protection against infections. However, a more specific immune response is also generated for a tailored response against specific infections and for immune memory. Based on the nature of response, the immune system can be segregated into two main types. This includes the innate and the adaptive immune responses.

3.1 Innate immunity

The innate immunity provides immediate protection and is therefore called the body's first line of defense (Netea et al., 2017). This is essential for example in case of wounding of the skin or upon entry of a pathogen. To combat this, the immune cells including the macrophages, mast cells, neutrophils, eosinophils, basophils, natural killer cells, and dendritic cells are recruited to site of wound/pathogen entry (Ganz, 2003; Dranoff, 2004; Netea et al., 2017). Upon injury or inflammation, the damaged cells of the tissues and tissue-resident macrophages produce cytokines and other inflammation associated molecules (Trepat et al., 2012; Netea et al., 2017). This causes the BECs, of the nearby blood capillaries, to upregulate their repertoire of cell surface proteins for example cell adhesion molecules like ICAM and VCAM (Ley et al., 2007; Nourshargh & Alon, 2014). This helps the leukocytes present in the blood flow to be attracted to the blood vessel walls and find the site of transmigration and eventually extravasate out (see section 4). These cells are then recruited to the site of wound or inflammation and an immediate immune response is generated.

The innate immune response works by the recognition of evolutionarily conserved molecules, including, lipopolysaccharides, dsRNA, peptidoglycans etc. (Vivier & Malissen, 2005). Some specificity in pathogen recognition is provided by pathogen associated molecular patterns (PAMP) and danger-associated molecular patterns (DAMP). Such pattern recognition patters can also be used to differentiate between the gram positive and negative bacteria (Medzhitov & Janeway, 2000; Netea et al., 2017). Nonetheless, if the immediate immune response fails to resolve the infection, a more specific immune response is required, i.e. the adaptive immune response.

Macrophages and DCs, amongst many other leukocyte sub-types, are the major cells of the innate immunity. However, due to their inherent ability to present antigens to other cells, they are exclusively essential in forming the bridge between the innate and

adaptive immune responses in the body (Banchereau & Steinman, 1998). In the context of adaptive immunity, the DCs represent the major type of leukocytes which engulf the antigens and migrate to the lymph nodes where they activate the T-lymphocytes, resulting in the initiation of the secondary (adaptive) immune response.

3.2 Dendritic cells and their role in adaptive immunity

Dendritic cells were first identified by Cohen and Steinman (Steinman & Cohn, 1973). They constitute the professional antigen-presenting subset of leukocytes and are continuously transported by the blood circulatory system. In homeostasis and upon inflammation, the circulating DCs extravasate the blood vessels and traverse deep within the surrounding tissues and the interstitial space. However, while some of the DCs actively patrol the tissues (migratory DCs), others become resident in certain organs like the thymus, spleen as well as the lymph nodes (tissue-resident DCs) (Vremec et al., 2000; Henri et al., 2001). DCs can originate from the lymphoid and myeloid progenitor (Figure 2). The tissue-resident lymphoid-derived DCs localize in the T-cell rich zones of the spleen and lymph nodes, whereas the myeloid-derived tissue-resident DCs are present in the marginal zone of the spleen (Zanna et al., 2021). Therefore, a variety of DCs exist in the body, allowing them to attain specific characteristics based on their localization and role in immunity. The DCs can be classified into conventional DCs (cDC1 and cDC2), plasmacytoid DCs (pDC), and Langerhans cells (LC). However, upon inflammation, monocyte can also differentiate into inflammatory DCs (Segura & Amigorena, 2013; Balan et al., 2019). The pDCs and LCs are most probably derived from the lymphoid origin (Anjuere et al., 2000; Collin & Bigley, 2018; Dress et al., 2019). All other types of DCs, regardless of their myeloid or lymphoid lineage, are generally called cDCs (Merad et al., 2013). However, it is generally believed that most of the classical cDCs originate from the myeloid origin via the common dendritic cell progenitor (Guilliams et al., 2014; Sichien et al., 2017) (see figure 2).

Apart from the lineage specific transcription factor-based identity of DCs, many surface markers are used to differentiate between different DC sub-types. These include, integrins CD1c, CD11b, CD11c, CD8α, CD4, major histocompatibility complex (MHC class-II), chemokine receptor XCR1, Langerin (in Langerhans DCs), and PDCA-1 (plasmacytoid dendritic cell marker) (Valladeau et al., 2000; Vremec et al., 2000; Henri et al., 2001; Bierly et al., 2008). Based on their protein signature, cDCs are normally MHC-II positive and express high level of CD11c (MHC-II^{+} CD11c^{hi}) (Alvarez, 2008). A previous study further described cDC1 subset as the CD141^{+} population and the cDC2 as the CD1c^{+} population of cDCs (Guilliams et al., 2014). More recently, the subsets of dermal cDCs have been reported to comprise of XCR1^{+} cDC1, XCR1^{-} CD11b^{+} cDC2 and XCR1^{-} CD11b^{-} cDCs (Kuroishi et al., 2020). However, based on numerous

other cDC specific markers, many other classifications can be drawn and therefore it remains to be a complex topic (Merad et al., 2013; McGovern et al., 2015; Schlitzer et al., 2015). The pDCs, which are essential in innate immunity, are known for the production of interferon-alpha (IFN-α), express low levels of MHC-II and CD11c (MHC-II$^{+/lo}$ CD11c^{lo}) (Alvarez, 2008). On the other hand, the Langerhans cells are mostly present in the epidermis and are known by their distinctive expression of langerin (CD207) and CD1a (Mizumoto & Takashima, 2004).

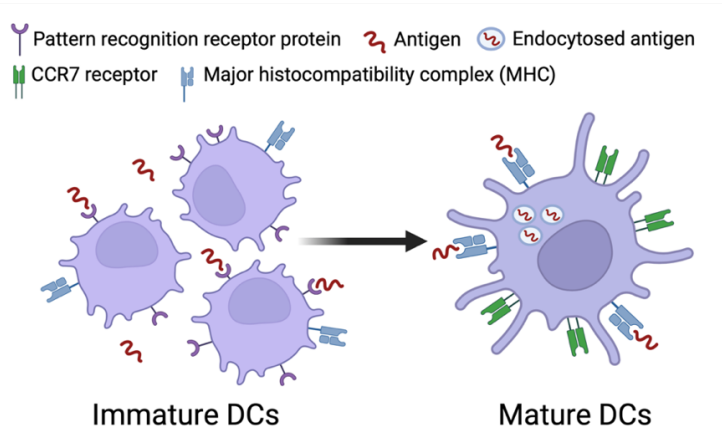


Figure 4: Dendritic cell maturation. Schematic shows DC maturation process. Immature DCs recognize the antigens via their pattern recognition receptors present on the DC surface (Gordon, 2002). The detected antigens are endocytosed. This induces the maturation of DCs, which is marked by an increase in the expression of major histocompatibility complex (MHC), which is needed for the surface presentation of the engulfed antigens. Additionally, mature DCs also upregulate their expression of several other cell surface proteins including CCR7 (Dieu et al., 1998; Sallusto et al., 1998), which is needed for the recognition of chemokine CCL21 and migration towards the lymphatics (Ohl et al., 2004). The schematic was created using BioRender.com.

DCs have a rough outer surface with long membrane protrusions or arms, called "dendrites" (Steinman & Cohn, 1973) (Figure 4). These dendrites in turn are essential in monitoring the environment and for searching host and foreign material. This is possible because the DCs express pattern recognition receptor protein which helps in the detection of host and foreign proteins (Gordon, 2002). Detection of these molecules, especially from a foreign pathogen, can trigger DC maturation (Figure 4). Upon engulfing host and foreign material, the DCs tend to display them on their surface as antigens (Dieu et al., 1998; Sallusto et al., 1998). To that end, they upregulate the expression of MHC (major histocompatibility complex) and co-stimulatory molecules, (Sallusto et al., 1995; Ueno et al., 2007). Additionally, as a part of the maturation process, the DCs also upregulate their expression of CCR7, which allows the guidance of DCs to the initial lymphatics (Sallusto et al., 1998; Yoshida et

al., 1998; MartIn-Fontecha et al., 2003; Aspelund et al., 2016; Schineis et al., 2019). Once inside the lymphatic vessel, the antigen-presenting DCs arrive in the lymph node. The lymph nodes have T-cell and B-cell rich areas, which are the main cells of the adaptive immunity (Dranoff, 2004). Here, the antigen presenting DCs, present their antigens to the T-lymphocytes, which starts the adaptive immune response (Forster et al., 2012). After their activation, the T-cells in turn activate the B-cells which produce antibodies (Kupfer et al., 1994; Garside et al., 1998). The soluble antigens can also reach the lymph nodes via the flow of the lymph. This can be taken up by the lymph node-resident follicular DCs, for presentation to the T-cells (Maurer et al., 2002; Sixt et al., 2005). The soluble antigens can also directly activate the B-cells and $CD4^+$ T-cells, which can interact with each other to boost their activation (Rastogi et al., 2022). This process could occur even before the antigen-presenting migratory DCs arrive to the lymph nodes (Pior et al., 1999; Ingulli et al., 2002; Itano et al., 2003). Therefore, the tissue resident DCs are also essential for the adaptive immunity.

A cohort of cDCs, present in the connective tissue of the dermis, which is responsible for active immune surveillance and adaptive immunity, are the dermal or interstitial DCs. Another type of DCs are the LCs, which patrol the epidermal tissue (Banchereau & Steinman, 1998; Merad et al., 2008; Helft et al., 2010). Existing evidence shows that the dermal DCs arrive in the draining lymph nodes more efficiently and faster than the LCs, and are therefore deemed as the major antigen presenting type of DCs (Kissenpfennig et al., 2005; Shklovskaya et al., 2008). Though dermal DCs are fewer in number as compared to the LCs, they are better at activating the T-cells in the lymph nodes (Helft et al., 2010).

In short, many types of types of leukocytes work together, via the adaptive and the innate immunity, to protect the host body. The purpose of both the innate and adaptive arms of the immune system is to provide defense against pathogens and to clear infections. Despite the common goal, both responses employ specialized group of cells and display unique molecular signatures which provides tight regulatory control.

4. Leukocyte extravasation from the blood vessels

One thing common in both the adaptive and innate immune responses, is the changing of compartments by the leukocytes. Although leukocytes exit the blood flow in homeostasis, their extravasation from the blood vessels, is greatly increased upon inflammation (Butcher, 1991; Springer, 1995; Aspelund et al., 2016; Schineis et al., 2019). As mentioned above, the extravasation of leukocytes from the blood vessels requires their diapedesis across the BEC barrier. Similarly, for the initiation of the adaptive immunity, their subsequent entry into the lymphatics requires crossing the LEC barrier. The role of endothelial cell barrier function is crucial in regulating the migration of leukocytes across the blood and lymphatic compartments and thereby affect the innate and adaptive immune responses in the body.

4.1 Importance of blood endothelial cell barrier

Beyond trafficking leukocytes, the blood comprises of plasma, water, and cells. Though the blood is confined in the circulatory system, active exchange of cells and solutes takes place between the blood vessels and the surrounding tissue (Jacob et al., 2016). Most of this exchange occurs at the endothelial cell junctions. Therefore, the blood endothelium serves as a checkpoint for the exchange of cells and solutes and is essential for maintaining homeostatic balance. The integrity of the endothelial cell junctions is maintained by cell adhesion proteins (Dejana et al., 2009). As described above, in the section on vessel architecture (see section 2.1), the permeability of the vasculature is maximum at the level of the capillaries, which provides the opportunity for effective exchange of cells and solutes. In this process, the cell adhesion molecules directly play an essential role in controlling the vascular permeability (Muller et al., 1993; Orsenigo et al., 2012). Some of the key adhesion proteins include VE-cadherin, junctional adhesion molecules (JAM), occludin, claudins, PECAM-1 etc. (Dejana et al., 2009). The cell junctions are dynamic in nature and allow the traffic of cells by opening momentarily and resealing. This dynamic activity requires constant endocytosis and recycling of junctional adhesion proteins and rearrangement of the actin cytoskeleton (Aghajanian et al., 2008). Numerous signaling pathways have been associated with opening of endothelial cell junctions and some of those are described in the sections below.

Inside the blood vessel, the BECs are directly exposed to the blood. On the abluminal side, however, the BECs connect to the basement membrane, pericytes and ECM, via focal adhesions, which are clusters of cell surface integrins (Marchand et al., 2019). These focal adhesions are essential extracellular sensors, detecting mechanical and chemical signals to help maintain vascular integrity through integrin binding with ECM components (Cohen & Guan, 2005).

Numerous diseases occur as a result of changes in vascular permeability, for example edema (Carmeliet & Jain, 2000; Dejana et al., 2009). Additionally, tumors also cause permeability changes and make the blood capillaries leaky due to underdeveloped endothelial cell junctions and loose connections with the surrounding pericytes (Hashizume et al., 2000; Morikawa et al., 2002). This leads to the leakage of growth factors and plasma into the tumor tissue matrix, further facilitating tumorigenesis and tumor related angiogenesis (Kumar et al., 2009). On the other hand, for leukocyte circulation between tissues, the extravasation of leukocytes from the blood vessels requires an increase in vascular permeability by modulating the cell junction adhesion proteins (Broermann et al., 2011). Therefore, it is absolutely essential to understand the processes which affect vascular permeability.

4.2 Mechanism of leukocyte transmigration across blood endothelia

Leukocyte egress from the blood vessels is a multi-step process requiring adhesion of leukocyte with the blood endothelial cells (BECs), on the luminal wall of the blood vessel. Most of the leukocyte extravasation occurs in the post-capillary venules, upon stimulation via inflammatory cues (e.g. histamine) or under the influence of cytokines (e.g. TNF and interleukins) (Pober & Sessa, 2007).

Upon local stimulation, the BECs express P-selectin and E-selectin, on the luminal surface of the blood vessel. The expression of these molecules is preferentially directed to the site from where the egress of leukocytes is needed i.e. the site of inflammation. The presentation of adhesion molecules at these sites, causes the freely travelling leukocytes in the blood, to be attracted towards the vessel wall and form adhesive interactions. This occurs due to the presence of P-selectin glycoprotein ligand-1 (PSGL-1), present on the leukocyte surface, which binds the BEC surface selectins (Ley et al., 2007). Once attached, the leukocytes can further attract other freely travelling leukocytes by binding leukocyte surface protein L-selectin with PSGL-1, peripheral node addressins (PNAd) or mucosal addressin cell adhesion molecule-1 (MadCAM), expressed on the surface of the attached leukocyte (Walcheck et al., 1996; Pendl et al., 2002; Grailer et al., 2009). The initial selectin based adhesive interactions between the leukocyte and blood endothelial cells are weak. However, they are enough to stimulate the leukocytes to roll over the vessel wall (Figure 5). The increased contact with the BECs, allows the leukocytes to bind endothelial produced chemokines (Rot & von Andrian, 2004; Lefort et al., 2012; Nourshargh & Alon, 2014). Chemokine binding of leukocytes in turn initiates signaling mediated by G-protein coupled receptor (GPCR), which activates leukocyte integrins (Zarbock et al., 2007).

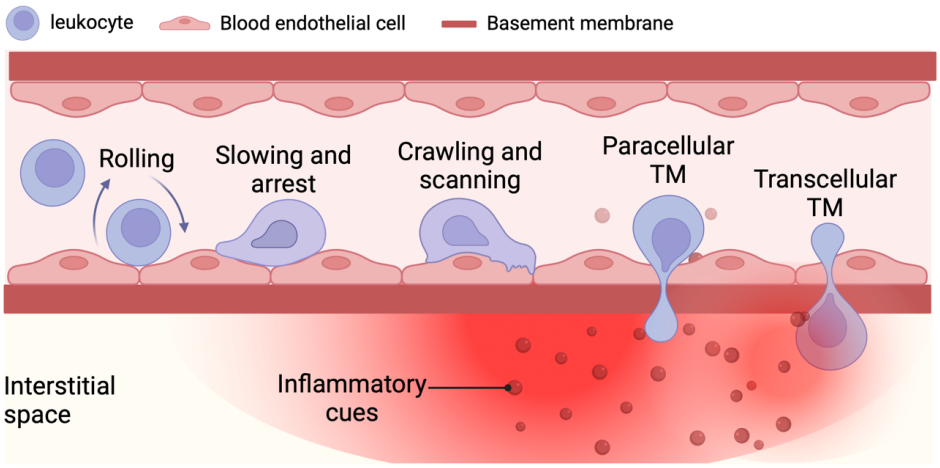


Figure 5: Extravasation of leukocytes from the blood vessels. The schematic summarizes the events related to the extravasation of leukocytes from the blood vessel. Upon sensing inflammatory cytokines, the blood endothelial cells (BECs), upregulate their expression of adhesive proteins on their luminal surface. This causes the attraction and rolling of leukocytes on the blood vessel wall. Eventually, more adhesive contacts develop which allow the spreading and arrest of leukocyte to the BEC surface. The attached leukocyte starts to crawl on the BEC surface and extends projections for scanning of the potential site of transmigration. Finally, the diapedesis event occurs either through the BEC junction (paracellular diapedesis) or through the BEC cell body (transcellular diapedesis). This schematic is modified from (Ley et al., 2007; Nourshargh and Alon., 2014). The schematic was created using BioRender.com.

The rolling leukocytes need further retarding force to slow down and finally arrest on the BEC surface (Figure 5). To do that the leukocytes extend microvilli projections in the form of rear membrane tethers (Schmidtke & Diamond, 2000; Ramachandran et al., 2004; Sundd et al., 2010) and forward slings (Sundd et al., 2012), over the BEC surface. These membrane protrusions have high expression of integrin LFA-1, which binds ICAM1 and ICAM2, present on the BEC surface (de Fougerolles & Springer, 1992; Xu et al., 1996; Sundd et al., 2012). Other integrins implicated in this process are VLA-4 binding to VCAM-1 and Mac-1 binding to ICAM1 and ICAM2 (Deckert-Schluter et al., 1994; Nourshargh & Alon, 2014). The increased interaction between the leukocyte and the BEC surface ultimately causes their arrest (Muller, 2013).

After the arrest, the leukocytes crawl on the surface of the blood vessel, against the blood flow, and explore the endothelial cell surface (Phillipson et al., 2009). The crawling leukocyte displays integrin mediated contacts, which protrude like arms, into the blood endothelia (Figure 5) (Shulman et al., 2009). This helps in firm binding to the BEC surface, and allows the leukocyte to crawl (Ward, 2009). The interaction

between the leukocyte and the BEC surface integrins activates Vav-1, which can regulate the reorganization of actin cytoskeleton (Mor et al., 2007; Phillipson et al., 2009). During the upstream crawling, using the modulation of actin cytoskeleton, the leukocytes engage their contractility machinery and display active millipede-like movement (Ward, 2009).

Previously, Caraman and Springer 2004, reported that during transcellular migration of leukocytes, a transmigration tunnel is formed through the BEC body, where LFA-1 and Mac-1 are richly expressed. Additionally, the BECs extend ICAM-1/VCAM-1 rich micro projections which, form a cup around the transmigration tunnel and, hug the transmigrating leukocyte (Carman & Springer, 2004). At the same time, the leukocytes, upon identification of a prospective transmigration site, extend a protrusive leading edge (uropod) through the transmigration tunnel across the BEC body (in transcellular diapedesis) (Hyun et al., 2012). This leads the way for the rest of the cell body to breach the BEC barrier (Figure 5). A previous study indicated that treatment with BAPTA-AM, colchicine, cytochalasin D or toxin-B considerably ablated monocyte transmigration across endothelial cell monolayer, as it hindered the formation of ICAM-1 projections at the transmigration site (Carman et al., 2003; Carman & Springer, 2004). Similarly, during paracellular diapedesis, upon the identification of a transmigration site, the leukocytes (monocytes), extend uropod through the BEC junctions which creates a path for their transmigration (Schenkel et al., 2002).

Leukocyte extravasation from the blood vessels also occur in the absence of inflammatory cues. Owing to their short life, the DCs are constantly replenished in the peripheral tissue, in steady state. To do that, immature DCs highly express C-type lectin DC-specific ICAM-3-grabbing non-integrin molecule (DC-SIGN), which binds ICAM3 (Geijtenbeek, Torensma, et al., 2000) and ICAM-2 (Geijtenbeek, Krooshoop, et al., 2000). Since, BECs highly express ICAM2 (Nortamo et al., 1991), which is similar in sequence to ICAM3 (de Fougerolles et al., 1993), it is more essential in the context of DC migration. Interestingly, Geijtenbeek et al., 2000, showed that immature DCs, breach the endothelia using ICAM2/DC-SIGN mediated rolling and binding mechanisms (Geijtenbeek, Krooshoop, et al., 2000). Additionally, it has been reported that in the steady state, the transendothelial migration of immature DCs is independent of β2-integrin mediated interactions, involving ICAM-1, VCAM-1 (Wethmar et al., 2006) and therefore LFA-1, Mac-1, VLA-4 are not needed in this process. Conclusively, leukocyte diapedesis can be orchestrated via several different mechanisms and across two distinct transendothelial cell routes (paracellular and transcellular). Many factors like leukocyte size, adhesiveness of BEC junctions and inflammation affect these routes.

Ultimately, the last step of the leukocyte diapedesis is the detachment of the leukocyte from the blood vessel surface on the abluminal side. This occurs when the integrins LFA-1 and VLA-4 are recycled, from the leukocyte-BEC contact site, to the leading edge of the leukocyte (i.e., on the side which is not in contact with the BEC surface). This breaks the integrin-mediated interactions between the leukocyte and the abluminal surface of the post-capillary venule. At the same time new ICAM-1, VCAM-1 mediated interactions develop between the leukocytes and the pericytes in the basement membrane, surrounding the venular wall (Nourshargh & Alon, 2014). These interactions facilitate the detachment of leukocytes from the blood vessel surface (Katagiri et al., 2006; Hyun et al., 2012; Nourshargh & Alon, 2014). At this point, the leukocytes sense any chemotactic cues (CXCL1, CXCL8, CCL2, MIF, IL-6), presented by the pericytes, which guides their transmigration across them and help their continued migration into the interstitial space (Stark et al., 2013; Nourshargh & Alon, 2014). The expression of some of these factors in pericytes, like ICAM-1 and MIF, are increased upon inflammation and, facilitate leukocyte egress and migration (Stark et al., 2013).

After extravasating from the blood vessels, the leukocytes navigate through the surrounding tissues where they encounter ECM components: fibronectins, laminin and collagen molecules. To navigate their way through this complicated network, the leukocytes express and secrete proteins which help with their mobility. For example, after the extravasation, the DCs upregulate the expression of matrix metalloproteases (MMP-2 and MMP-9), which assists their migration through the tissue matrix (Ratzinger et al., 2002). In turn, the tissues produce tissue inhibitor of metalloproteinases (TIMPs), which restricts DC migration. To migrate, DCs must overcome this challenge by increasing the production of MMPs (Ratzinger et al., 2002; Darmanin et al., 2007). Additionally, expression of adhesion molecules on leukocyte surface also helps the mobility of leukocytes in the tissues.

4.3 Hotspots of leukocyte migration in blood endothelia

Approximately 90% of transendothelial leukocyte migration, in the blood vessels, occurs via the paracellular route i.e. through the blood endothelial junctions (Woodfin et al., 2011). However, depending on their size, smaller leukocytes like T-cells can also transmigrate through the cell body, i.e. the transcellular route (Krug et al., 2009; Filippi, 2016). Unlike the post capillary venules, the brain vasculature is comparatively harder to breach, due to the presence of stronger adhesive interactions between the BECs (Wolburg et al., 2005). This reduces the permeability of the blood vessels and helps to maintain the integrity of the blood-brain barrier (Dejana, 2004). Due to the less permeable junctions in the brain vasculature, transcellular transmigration route is favored over the paracellular transmigration (Nourshargh & Alon, 2014). Other than

in brain, only a small number of leukocytes use the transcellular path of diapedesis (Engelhardt & Ransohoff, 2012).

Interestingly, in the post capillary venules, about 75% of the leukocyte transmigration occurred through the tri-cellular junctions (where boundaries of 3 adjacent cells meet) (Burns et al., 1997). This was attributed to the low-density and discontinuous expression of cell junction adhesion proteins at these sites, creating a path of least resistance that favors leukocyte diapedesis (Burns et al., 1997). Other studies have also reported similar observations about the existence of preferential diapedesis through the tri-cellular junctions as opposed to the bi-cellular junctions (where boundaries of 2 adjacent cells meet) (see Figure 6) (Sumagin & Sarelius, 2010). Additionally, Hyun et al., 2019 reported that multiple neutrophils transmigrate across the blood endothelia, using the same site (Hyun et al., 2019). This means that the first diapedesis event somehow conditions the transmigration site which marks it for future detection and transmigration by the leukocytes. The presence of these "hotspots" of leukocyte diapedesis is astonishing and points towards the special architecture of the BEC tri-cellular junctions as compared to the bi-cellular junctions. This is discussed further in section 4.6 below.

4.4 Architecture of blood endothelial cell junctions

Adjacent BECs, arrange laterally to form cell-cell adhesive connections, enabling the formation of a monolayer. In doing so, the endothelial cells display bicellular junctions (shoulder to shoulder contact between two adjacent cells), and multicellular junctions (vertices where more than two cells meet) (Figure 6). These adhesive contacts originate due the presence of cell adhesion proteins, which ensure the sealing of the cell junctions and therefore stop leakage, as explained in section 4.1.

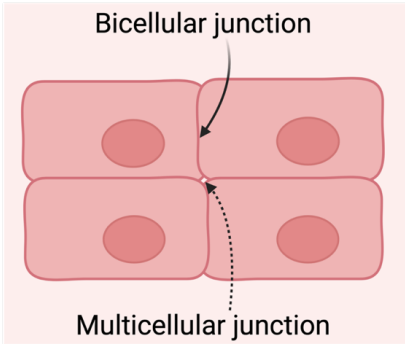


Figure 6: Bicellular and multicellular junctions. The schematic shows bicellular junctions (cell junction contact between two adjacent cells) and multicellular junction

(where cell junctions of multiple adjacent cells meet). The schematic was created using BioRender.com.

The blood endothelial barrier comprises of the two main types of cell junctions– the tight junctions and the relatively weaker adherens junctions. Each of these types of junctions are distinguished by their unique molecular structure and strength, which enables them to collectively regulate leukocyte trafficking and exchange of cells and solutes, across the endothelium (Hartsock & Nelson, 2008). The primary distinction between the two types of blood endothelial cell junctions lies in their unique repertoire of adhesion proteins, each characteristic of a specific junction type (Figure 7). These are described below.

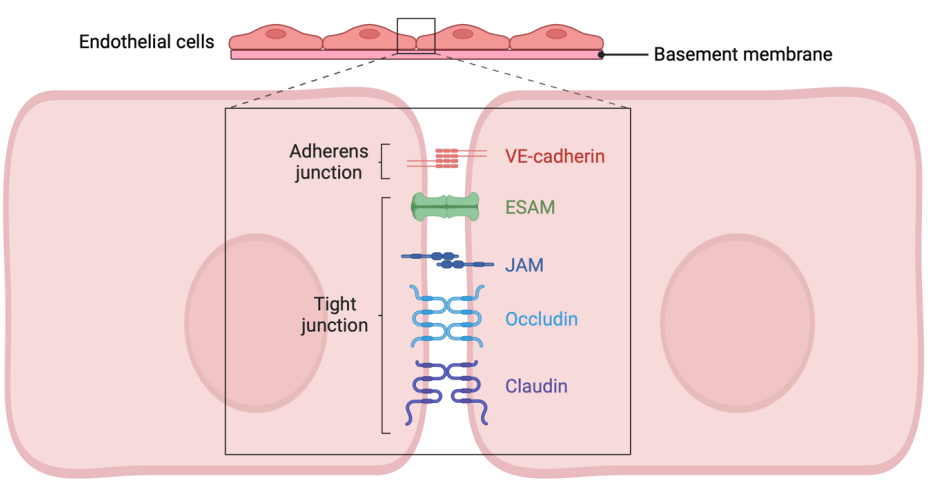


Figure 7: Types of cell junction adhesion. The schematic shows cell adhesion proteins responsible for adherens versus the tight junctions. These molecules include claudin, occludins, Junctional adhesion molecule (JAM), Endothelial cell selective adhesion molecule (ESAM) and Vascular endothelial cadherin (VE-cadherin) (Dejana, 2004; Hartsock & Nelson, 2008; Nourshargh & Alon, 2014; Trepat et al., 2012). This schematic is modified from (Zhao et al., 2022). The schematic was created using BioRender.com.

4.4.1 Tight junctions

Tight junctions are normally ascribed as the “fence” and “gate” between different compartments. In doing so, they control the mixing of cellular proteins for example, between the apical and basal sides of the membrane, in epithelial cells (Anderson & Van Itallie, 2009). Tight junctions are also present in endothelial cells where due to their stringent sealing strength; they provide an excellent barrier to the paracellular exchange of solutes between the cells. The strength of the tight junctions in the blood

vessels could be different depending on their location. For example, the tight junctions present in the blood brain barrier are the strongest (Claesson-Welsh et al., 2021).

The junctional cell adhesion proteins associated with the tight junctions include claudin, occludin, JAMs, endothelial cell selective adhesion molecule (ESAM) and the cytoplasmic Zonula occludens proteins ZO1, ZO2 and ZO3 (Figure 7) (Dejana, 2004; Hartsock & Nelson, 2008; Trepat et al., 2012; Nourshargh & Alon, 2014). Another essential protein associated with tight junctions, present in the blood brain barrier and in epithelial cells, is tricellulin (Ikenouchi et al., 2005; Iwamoto et al., 2014; Cho et al., 2022). Interestingly, in the brain vasculature, the BEC tri-cellular junctions show a relatively higher expression of tricellulin as compared to the bi-cellular junctions (Ikenouchi et al., 2005; Krug et al., 2009). This extra reinforcement could stabilize the cell junctions and thereby limit the permeability of the blood brain barrier.

4.4.2 Adherens junctions

Conversely, the adherens junctions are important in providing the first adhesive interactions between the cells. Additionally, they also affect the regulation of intracellular signaling, via their association with the actin cytoskeleton machinery, and provides transcriptional control (Hartsock & Nelson, 2008). Unlike the tight junctions, the main adhesion molecule of the adherens junctions is the vascular endothelial (VE)-cadherin, which via its interaction with β-catenin, α-catenin and p120-catenin, connects the cell junctions to the cell cytoskeleton (Hartsock & Nelson, 2008; Trepat et al., 2012).

Cadherin is a transmembrane protein with 5 extracellular domains (EC1-5). The EC1 domain of adjacent cells form homodimers, by binding to calcium ions, and provide adhesion between cells (Nagar et al., 1996). The cytoplasmic part of the cadherin protein is composed of two sub-domains. One of which is closer to the plasma membrane and binds to p120-catenin (Yap et al., 1998; Iyer et al., 2004; Dejana et al., 2009) and the other is extended more inside the cytoplasm and binds β-catenin (Hartsock & Nelson, 2008; Dejana et al., 2009; Trepat et al., 2012). It was believed that the VE-cadherin/β-catenin complex can be connected to actin cytoskeleton via α-catenin (Aberle et al., 1994; Knudsen et al., 1995; Rimm et al., 1995). However, more recent evidence negated the existence of VE-cadherin/β-catenin/α-catenin/actin, co-existing in a single complex (Drees et al., 2005; Yamada et al., 2005). In the light of these evidence, the common understanding in the field was that certain other proteins might be essential in connecting the VE-cadherin/β-catenin complex to the α-catenin/actin complex (Trepat et al., 2012). In epithelial cells for example, an actin-binding protein called Lima-1 or EPLIN (epithelial protein lost in neoplasm), was shown to link cadherin-catenin complex to F-actin (Abe & Takeichi, 2008). However,

in endothelial cells, other proteins called AmotL2 and MAGI1 have been shown to connect junctional VE-cadherin to contractile actin fibers (Hultin et al., 2014; Zhang et al., 2023). VE-cadherin has been implicated in a variety of cell signaling pathways which control vascular permeability. The connection of junctional VE-cadherin with the actin cytoskeleton shows how modulation of VE-cadherin junctions might affect the contractility in endothelial cells, especially during the transmigration event (see section 4.5).

The adhesive strength of adherens junctions is affected by various signaling pathways such as tyrosine kinases (HGF and EGFR) and non-tyrosine kinase receptors (SRC, ABL, EPH) (Gumbiner, 2005; Trepat et al., 2012). Based on the existing evidence it can be envisioned that the modulation of VE-cadherin mediated junctional adhesions is crucial for paracellular migration of leukocytes out of blood vessels (Nourshargh & Alon, 2014; Vestweber, 2015) and into the lymphatic vessels, which is a pre-step for the activation of adaptive immunity (Forster et al., 2012; Schineis et al., 2019).

4.5 Mechanism of blood endothelial cell junction opening

BEC junctions are intricately decorated with a variety of cell junction adhesion molecules which prevents leakiness, as described in the section above (section 4.1). Nonetheless, trafficking of solutes and cells mostly occurs through BEC junctions, which requires their opening and resealing. The process of cell junction maintenance is dynamic and involves the endocytosis and recycling of cell junction adhesion proteins and active reorganization of cell cytoskeleton. An important junctional adhesion protein in this context is VE-cadherin. As described below (see section 4.5.1), the lateral border recycling compartment (LBRC) might play an essential role in the recycling of the junctional proteins. However, VE-cadherin is not recycled via the LRBC (Mamdouh et al., 2009). Nevertheless, previous studies show that VE-cadherin rearrangement and recycling is necessary for the opening of cell junctions and therefore leukocyte diapedesis (Broermann et al., 2011; Wessel et al., 2014). The mechanisms associated with the VE-cadherin mediated opening of blood endothelial cell junctions are described below (Figure 8).

4.5.1 Calcium (Ca^{+2}) dependent mechanisms

In blood endothelia, the adherens junction protein VE-cadherin disassembles and allows the opening of the cell junction during a diapedesis event (Nourshargh & Alon, 2014; Vestweber, 2015). The exact mechanism of VE-cadherin disassembly mediated junction opening is not known but there are two widely accepted hypotheses in the field. According to the first hypothesis, the VE-cadherin disassembly can be mediated

by thrombin, produced as a result of tissue injury. This stimulates the post capillary venules in the intact tissue and causes the remodeling of endothelial cell cytoskeleton and the creation of small pores between endothelial cells, which in turn causes an influx of calcium ions. The elevated intracellular Ca^{+2} leads to the activation of calcium-calmodulin-dependent myosin light chain kinase and small GTPase RhoA, which in turn causes the phosphorylation of myosin light chain and modelling of F-actin stress fibers with the activation of Rho associated protein kinase (ROCK) (Carbajal et al., 2000; Muller, 2003). Ultimately, this causes the phosphorylation of VE-cadherin/catenin complex and together with the rearrangement of cellular actin, causes the retraction of endothelial cell and therefore the opening of cell junction and the transmigration of leukocyte (van Nieuw Amerongen et al., 2000; Goswami & Vestweber, 2016; Schimmel et al., 2017) (Figure 8).

On the other hand, leukocyte arrest on the blood vessel wall can also induce an increase in the intracellular Ca^{+2} level of the blood endothelial cell (Huang et al., 1993). This induces the same myosin light chain kinase and RhoA mediated opening of VE-cadherin junctions however, this is a more local effect as compared to that caused by thrombin. Additionally, during an active diapedesis event, the transmigrating leukocyte also exerts a mechanical push on the endothelial cell junctional VE-cadherin, which causes its displacement, allowing a path for leukocyte transmigration. After the transmigration, the junctional VE-cadherin is restored which causes resealing of the junction (Shaw et al., 2001).

Similar to VE-cadherin, another protein present at the endothelial cell junctions is PECAM-1 (Privratsky & Newman, 2014). During a transmigration event, the increased calcium signaling inside the BEC also affects the PECAM1 mediated adhesion between cells. One study had previously reported that upon diapedesis, PECAM1 and its partner TRPC6 (calcium channel) surround the site of transmigration. Interestingly, inhibiting TRPC6, causes the failure of the transmigration event (Weber et al., 2015). This shows that a cohort of cell adhesion proteins and their co-partners are essential in orchestrating a successful diapedesis event.

TRPC6 is also needed in the recycling of LRBC, which is essential in the diapedesis process (Weber et al., 2015). Previous studies have indicated that the LRBC is recycled at the site of diapedesis, to provide extra membrane and molecular machinery which supports the diapedesis process and limits the leakage of cytoplasm (Vestweber, 2015). Cell junction proteins like PECAM-1, JAM-A and CD-99 etc. are constantly exchanged between the LBRC and the cell junctions (Muller, 2011). This helps in maintaining the integrity of the cell junctions during the diapedesis event. It is known that the treatment of cells using blocking antibody against JAM or PECAM1, ablates monocyte and neutrophil transmigration (Muller et al., 1993; Martin-Padura et al., 1998; Del Maschio et al., 1999).

4.5.2 Calcium (Ca^{+2}) independent mechanisms

The second hypothesis proposes that vascular permeability inducing factors like histamine and vascular endothelial growth factor (VEGF-A) can also mediate the opening of the VE-cadherin junction (Dejana et al., 2008; Nottebaum et al., 2008). Endothelial cells can sense VEGF-A by their surface VEGF receptor (VEGFR2). This, via downstream signaling, leads to the activation of focal adhesion kinase (FAK) (Abedi & Zachary, 1997), which binds integrins and VE-cadherin (Chen et al., 2001; Chen et al., 2012). FAK in turn mediates the phosphorylation of β-catenin, which causes the breakdown of β-catenin-VE-cadherin complex. This causes the opening of the endothelial cell junctions. Preventing FAK activation, hinders the opening of the endothelial cell junctions (Chen et al., 2012).

Alternatively, previous reports have showed that the interaction of VE-cadherin with vascular endothelial protein tyrosine phosphatase (VE-PTP) is essential for the integrity of the VE-cadherin junction (Nawroth et al., 2002; Nottebaum et al., 2008). A report showed that VE-PTP stabilizes the VE-cadherin junctions by inhibiting the Rho GEF (GEF-H1), which prevents the activation of RhoA and the downstream modulation of junctional VE-cadherin junctions (Juettner et al., 2019). Dismantling the interaction between VE-cadherin and VE-PTP leads to the opening of cell junctions while stabilizing VE-cadherin/VE-PTP interactions hinders junction opening and also the associated paracellular leukocyte transmigration (Broermann et al., 2011). To support its function, VE-PTP requires interaction with the VE-cadherin-plakoglobin complex. The β-catenin was not found to be essential in this process (Nottebaum et al., 2008) (Figure 8). Nottebaum et al., also reported that VEGF-A/VEGFR2 signaling can cause the disruption of the VE-cadherin/VE-PTP complex and induce tyrosine phosphorylation of the β-catenin. This could then lead to the opening of the endothelial cell junctions, as explained above. Additionally, a study showed that leukocyte interaction with endothelial cells VCAM-1 can cause the detachment of VE-PTP from VE-cadherin, which can also result in the opening of the endothelial cell junction (Vockel & Vestweber, 2013). Therefore, dismantling of the VE-cadherin/VE-PTP complex can occur via numerous ways.

However, the question that remains is whether the dissociation of VE-PTP causes endocytosis of VE-cadherin which ultimately leads to the opening of the cell junctions? A study reported that the disruption of VE-cadherin/VE-PTP complex does not trigger the endocytosis of VE-cadherin (Nourshargh & Alon, 2014). Nonetheless, other reports have indicated that leukocyte-BEC contact can induce the dissociation of tyrosine kinase SHP2 which can lead to the dephosphorylation of VE-cadherin at its tyrosine residue (tyr731) (Arif et al., 2021). This can further induce the recruitment of adaptin AP-2 protein and lead to the endocytosis of VE-cadherin (Wessel et al., 2014). The endocytosis of junctional VE-cadherin can therefore weaken the cell junction

integrity and cause the opening of cell junctions and allow leukocyte transmigration. Alternatively, leukocyte interaction with the endothelial cells can trigger the rearrangement of junctional VE-cadherin, which generates de novo gaps in junctional VE-cadherin, and thus favor leukocyte diapedesis (Shaw et al., 2001).

Furthermore, previous studies showed that VCAM-1 and ICAM-1 mediated binding of leukocyte to the endothelial cell surface can activate NADPH oxidase and nitric oxide synthase (eNOS) (Matheny et al., 2000; Deem et al., 2007; Martinelli et al., 2009). These oxidases generate reactive oxygen species which triggers the activation of protein kinase C (PKC) and matrix metalloproteinases (MMPs), which assist in opening of the endothelial junctions (Matheny et al., 2000; Deem et al., 2007; Turowski et al., 2008; Martinelli et al., 2009). This could occur either via PKC mediated activation of Src kinase and small GTPase Rho, which in turn affect the rearrangement of actin cytoskeleton (DerMardirossian et al., 2006; Turowski et al., 2008), or via the MMP mediated disruption of junctional adhesion proteins, as shown for the disruption of tight junctions in the brain (Y. Yang et al., 2007).

Therefore, leukocyte-blood endothelial cell interaction can mediate the opening of endothelial cell junctions via a variety of different routes (Figure 8). It is possible that prior to the transmigration event, the dissociation of junctional VE-cadherin/catenin complex provides the initial weakening of the cell junctions by engaging the contractility machinery and creating a pull at the junctions. However, during the transmigration event, some of VE-cadherin is internalized, which creates the transmigration tunnel, through which the transmigration occurs. It is therefore possible that the calcium dependent and calcium independent routes of VE-cadherin modulation are not mutually exclusive but occur alongside each other, to trigger the opening of the blood endothelial cell junctions. Moreover, co-operation of these routes could at the same time provide additional control in limiting the leakage of vessels, during the transmigration process.

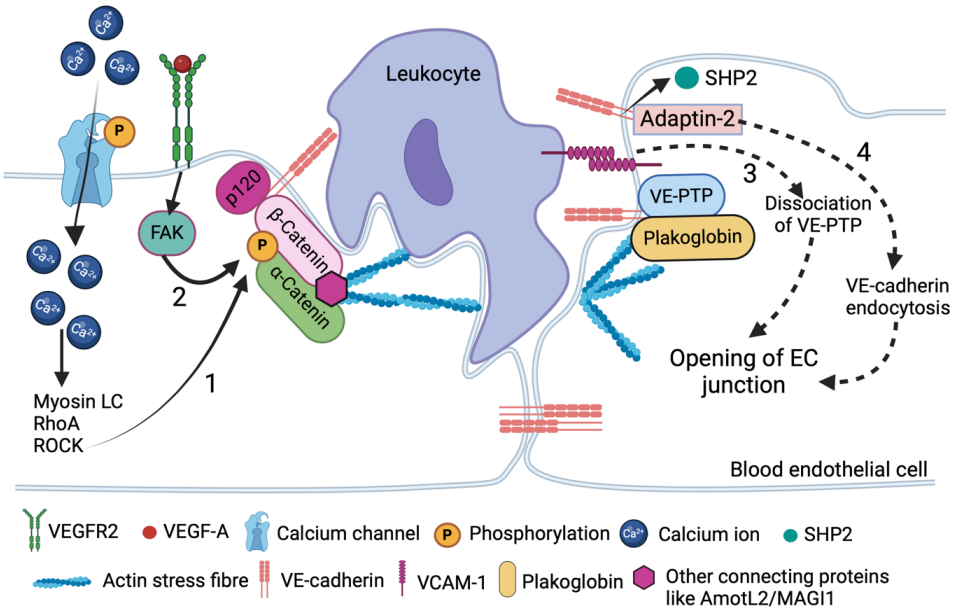


Figure 8: Endothelial junction opening via the modulation of VE-cadherin. The schematic summarizes different ways which might usher the opening of the blood endothelial cell (BEC) junctions, via the disruption of junctional VE-cadherin. (1) Tissue injury, leukocyte-BEC contact, or the effect of thrombin can cause the influx of calcium ions into the BECs. This causes the phosphorylation of myosin light chain via RhoA and subsequent remodeling of actin fibers under the influence of Rho associated kinase (ROCK). This induces cell contractility, and the phosphorylation of the VE-cadherin associated β-catenin, which ultimately causes the opening of the BEC-BEC junctions (Goswami and Vestweber, 2016; Carbajal et al., 2000; Muller, 2003; Schimmel et al., 2017; van Nieuw Amerongen et al., 2000; Huang et al., 1993). Alternatively, (2) VE-cadherin-catenin complex can be phosphorylated via the activation of focal adhesion kinase (FAK), upon the binding of VEGF-A to VEGFR2, present on the BEC surface (Abedi and Zachary, 1997). This causes the phosphorylation of β-catenin and causes its dissociation from the VE-cadherin. The disruption of this complex causes the opening of the VE-cadherin junction (Chen, 2012), possibly via the ROCK mediated activation of the cell contractility machinery. Moreover, (3) VEGF/VEGFR2 binding or homophilic interactions between the BEC and leukocyte surface VCAM1, can induce the dissociation of VE-PTP from the VE-cadherin and can trigger the opening of the BEC junction (Broermann et al., 2011; Vockel and Vestweber, 2013). Conversely, (4) leukocyte/BEC interaction can cause the dissociation of SHP-2 from the VE-cadherin and lead to the recruitment of adaptin-2 which can induce the endocytosis of junctional VE-cadherin and cause the BEC junction to open (Wessel et al., 2014). This schematic was modified from (Ley et al., 2007; Azzi et al., 2013; Nourshargh & Alon, 2014). The schematic was created using BioRender.com.

Conclusively, the process of junction opening and closing is tightly regulated by a host of molecular signaling mechanisms which include modulation of cell junction

adhesion molecules, actin myosin complex and the rearrangement of cell cytoskeleton (Huang et al., 1993; Hixenbaugh et al., 1997; Saito et al., 2002; Allingham et al., 2007). Modulation of the regulatory processes, which control cell junction opening, can regulate leukocyte transmigration (Saito et al., 2002; Allingham et al., 2007) and can thereby provide ways to control the extent of inflammatory responses in the body.

4.6 Guidance of leukocytes across blood endothelia

The blood endothelia, presents a transmigration barrier by intricately controlling the extent of leukocyte egress from the blood vessels. Previous reports have indicated that some BEC junctions (tri-cellular junctions) are preferred over the others for the transmigration of leukocytes (Burns et al., 1997; Wang et al., 2006; Gorina et al., 2014). Moreover, in some instances, the same transmigration site can be used by multiple leukocytes to exit the blood vessel, and represent a transmigration "hotspot" (Hyun et al., 2019). However, if the transmigration site is not repeatedly used by other leukocytes, but is generally chosen over other possible routes, then it is referred as "preferential site" in this thesis (Burns et al., 1997). Nonetheless, in both cases leukocytes are somehow first guided to the transmigration sites (Wang et al., 2006; Mydel et al., 2008; Lim et al., 2015; Girbl et al., 2018). Understanding the process which guide the migrating leukocytes to the site of transmigration, is therefore essential in controlling the extent of leukocyte transmigration.

A study previously described, that chemokine CXCL2 could be immobilized on BEC surface via heparan sulfate, which could guide the neutrophils to the site of inflammation (Massena et al., 2010). Intriguingly, a more recent study reported that infact upon induction of inflammation, the venular BECs, and the pericytes surrounding them, secrete chemokine CXCL1. This is sensed by the neutrophils, migrating inside the lumen of the venules, via their CXCR2 receptor (Girbl et al., 2018). The CXCR2/CXCL1 binding leads to the attaching of the neutrophil to the vessel wall. After the arrest, the neutrophil starts to produce chemokine CXCL2, while the interacting BECs upregulate their expression of atypical chemokine receptor 1 (ACKR1), which gets enriched at the BEC junctions (Girbl et al., 2018). ACKR1, binds CXCL2 but because it lacks the signaling motif, it only acts as a sink, which creates a reservoir of chemokine CXCL2 (Pruenster et al., 2009). The ACKR1/CXCL2 binding helps the neutrophils to probe the BEC junctions, in a self-guided manner (Girbl et al., 2018). However, it is still unclear what guides the neutrophils to the transmigration site, on the plane of the blood endothelial cells. Nonetheless, based on the existing data it can be believed that chemotactic cues might direct the migrating leukocytes to the transmigration site.

Furthermore, it can be envisioned that the transmigration site also presents specific cues to the arriving leukocyte. This could for example occur via a special molecular signature or "landmark", which differentiates the transmigration site from the rest of the endothelial cell surface. Previous studies have shown that the ICAM1 is enriched at the transmigration site, in the blood endothelia (Burns et al., 1997; Sumagin & Sarelius, 2010; Arts et al., 2021; Gronloh et al., 2023). This could help in the arrest of the leukocyte and act as a "landmark", which differentiates the preferential transmigration site, from the rest of the BEC junctions. It is already known that surface expression of integrins and cell adhesion molecules including PECAM1, ICAM1, ICAM2, JAMs, CD99 and endothelial cell selective adhesion molecule (ESAM) help the migrating leukocyte to navigate their surrounding environment (Nourshargh & Alon, 2014). Therefore, the interaction of leukocyte surface integrins with endothelial cell surface, could be envisioned to guide the leukocytes to the preferential transmigration sites.

Nonetheless, the guidance of migrating leukocyte does not end there. After exiting the blood vessel, to enter the tissues, the leukocytes need to still migrate across the extracellular matrix, basement membrane and pericytes. Interestingly, here as well the leukocytes are welcomed with pre-existing preferred routes of migration. Additionally, they follow the cues provided by the surrounding pericytes and the basement membrane architecture (Proebstl et al., 2012). The pericyte wall, just outside the blood endothelia, and the extracellular matrix proteins (collagen IV, nidogen-1, nidogen-2, perlecan, laminin 411 and laminin 511), arrange in a way which leaves some open gaps in between the cells. During inflammation these gaps can become wider, and the leukocytes are guided through these sites (Proebstl et al., 2012). Pericytes upon sensing inflammatory stimuli, increase their expression of ICAM-1 and VCAM-1 specifically at the site of inflammation. Together with this, the expression of laminin 511 collagen IV is reduced, which marks the preferential migration sites (Wang et al., 2006). Migrating leukocytes sense these changes and traverse towards the inflammation site using Mac-1, LFA-1, VLA-4 mediated pathways (Verbeek et al., 1995; Proebstl et al., 2012). In the light of these evidence, it can be envisioned that migrating leukocytes constantly monitor and interact with their surrounding microenvironment. The sensing of guidance cues, present in the vicinity helps in the directed migration of leukocytes.

5. Leukocyte transmigration in the lymphatic vasculature

Leukocyte migration into the lymphatics, is required for the functioning of the immune system (Forster et al., 2008; Schineis et al., 2019). However, to enter the lymphatic vessel lumen, the DCs must breach the LEC barrier. Since the lymphatic endothelium controls the exchange of solutes and cells between the lymphatic vessel and the surrounding tissues, it is essential to understand how this barrier is established and how is it compromised during the transmigration of DCs.

5.1 Architecture of lymphatic endothelial cell junctions

Similar to the BECs, the LECs, laterally arrange with adjacent LECs, forming bi-cellular junctions (where borders of two LECs meet) and multicellular junctions (where borders of more than two LECs meet). These LEC-LEC adhesive contacts are essential in determining the permeability of the lymphatics thereby, controlling the exchange of fluid and immune cells.

5.1.1 Lymphatic capillaries versus lymphatic collectors

The LECs of the lymphatic collector vessels have continuous “zipper-like” junctions, similar to the blood endothelia (Baluk et al., 2007; Baluk & McDonald, 2022) (Figure 9). Here, the continuous localization of cell adhesion proteins, strengthen the LEC junction integrity and make them comparatively harder to breach. Additionally, the collector vessels are reinforced with a thicker continuous basement membrane and contractile smooth muscle cells (Stritt et al., 2021) (Figure 9).

However, the lymphatic capillaries have a discontinuous basement membrane. Additionally, the LECs of the blind-ended lymphatic capillaries display unique characteristics, as compared to the lymphatic collectors (Pflicke & Sixt, 2009). They attain a distinct oak-leaf like shape and have discontinuous junctional VE-cadherin, which forms the “button-like” junctions (Baluk et al., 2007; Baluk & McDonald, 2022) (Figure 9). Upon inflammation however, the LEC button-like junctions transition to a zipper-like conformation, akin to the LECs in the lymphatic collectors (Yao et al., 2012). This plasticity of LEC junctions is essential in determining the permeability of the lymphatics (Zhang et al., 2020). One study proposed that in steady state, the overlapping membranes of the adjoining LECs in the lymphatic capillaries, represent flaps and play a role in controlling the flow of tissue fluid into the lymphatic vessel lumen (Baluk et al., 2007). Therefore, the lymphatic vessels exert control over what enters the lymphatic system possibly by modulating the LEC junctions.

5.1.2 Lymphatic endothelial cell junctional adhesion proteins

It has been shown that both button and zipper-like LEC junctions have VE-cadherin (Knudsen et al., 1995; Iyer et al., 2004; Dejana et al., 2009; Yao et al., 2012; Hultin et al., 2014; Baluk & McDonald, 2022). In BECs, the VE-cadherin was shown to be connected to the actin cytoskeleton via catenin and other associated molecules (Knudsen et al., 1995; Iyer et al., 2004; Dejana et al., 2009; Yao et al., 2012; Hultin et al., 2014; Zhang et al., 2023). Based on that it can be envisioned that, in LECs too, VE-cadherin can affect actin cytoskeleton and cell contractility machinery. Additionally, VE-cadherin has also been reported to function as a mechanotransduction sensor for the activation of lymphatic specific genes, during development (Yang et al., 2019). The VE-cadherin was reported to be essential in the development of lymphatic vessels (Hagerling et al., 2018), and the formation of valves (Yang et al., 2019), which prevents the back-flow of lymph, in the lymphatic collectors (Aspelund et al., 2016) (Figure 9).

Along with VE-cadherin, PECAM-1 and LYVE-1 are also present at the LEC junctions (Baluk et al., 2007; Tal et al., 2011; Yao et al., 2012). Unlike PECAM-1, which is present at both button and zipper junctions (Baluk et al., 2007), LYVE-1 is absent in the zipper junctions of the lymphatic collectors and larger lymphatic vessels (Johnson et al., 2017). Apart from that, some studies have also reported the occurrence of connexins – gap junction proteins – which are important in the development of lymphatic collector valves and thereby ensure, unidirectional flow of the lymph (Kanady et al., 2011; Sabine et al., 2012). Other tight junction proteins which have been attributed to LEC junctions, include ZO-1, claudin-5, occludin, JAM-A and ESAM (Baluk et al., 2007; Yao et al., 2012). Existing evidence shows that VE-cadherin does not affect the localization of LEC tight junction proteins, as its deletion does not alter the tight junction formation or organization (Baluk et al., 2007; Hagerling et al., 2018). JAM-A has been found to be essential in the extravasation of neutrophils from the blood vessels (Martin-Padura et al., 1998; Del Maschio et al., 1999). In lymphatics, JAM-A has been reported to control DC motility and trafficking to the lymph nodes. Previous study has reported that the deletion of JAM-A in DCs, increased the randomness in DC motility and their transmigration across the LECs. This ultimately also enhanced the homing of DCs to the lymph nodes (Cera et al., 2004). Therefore, it is possible that JAM-A mediated interactions between DCs and their microenvironment play essential role in their directed migration. Future studies to ascertain this are required.

Conclusively, VE-cadherin and other junction associated adhesion proteins maintain the integrity of the LEC junctions (Baluk et al., 2007). The discontinuity in the junctional adhesion proteins and the basement membrane, at LEC button junctions, possibly allow the easy entry of cells and tissue fluid from the interstitial tissue into the lymphatic vessels (Trzewik et al., 2001; Baluk et al., 2007). To support that, previous studies have shown that most of the DC transmigration, into the lymphatics,

occurs through the discontinuous junctions of the lymphatic capillaries (Pflicke & Sixt, 2009). However, DCs can also enter the lymphatic vessel lumen and reach the lymph nodes across the zipper-like junctions of the lymphatic collectors (Arasa et al., 2021) or upon transitioning of lymphatic capillary button-like junctions to zipper-like junctions, in inflammation (Yao et al., 2012).

Additionally, tumor cells can get directly integrated into the layer of lymphatic endothelium by penetrating through the adjacent LECs (Kerjaschki et al., 2011). Additionally, some studies have also reported that leukocytes and tumor cells might also transmigrate into the lymphatics via the transcellular path (through the cell body) (Azzali, 2006; Teijeira et al., 2013; Schimmel et al., 2017), although, it is not the preferred route of their transmigration. Similarly, evidence for transcellular exchange of solutes via clathrin or caveolae-mediated endocytosis also exists (Triacca et al., 2017). Another study reported that transcytosis of solutes (larger proteins like soluble antigen), can occur via dynamin dependent route in LECs present in the lymph node (Kahari et al., 2019). This shows that though the presence of LEC specialized button junctions allow efficient uptake of fluid and leukocytes, it is not the only way for the exchange of material.

5.2 Dendritic cell transmigration across lymphatic endothelia

Upon engulfing the antigens, the DCs mature and need to find their way to the lymphatics. This is achieved by the upregulation of numerous cell surface proteins including CCR7, which is the cognate receptor for chemokine CCL21 (Yoshida et al., 1998; MartIn-Fontecha et al., 2003). Using this, the DCs detect the chemokine CCL21, which is secreted by the initial lymphatics of the surrounding tissues and, also, inside the paracortex of the lymph nodes (Weber et al., 2013; Ulvmar et al., 2014). This allows the attraction of DCs towards the lymphatics (Figure 9) (Forster et al., 1999).

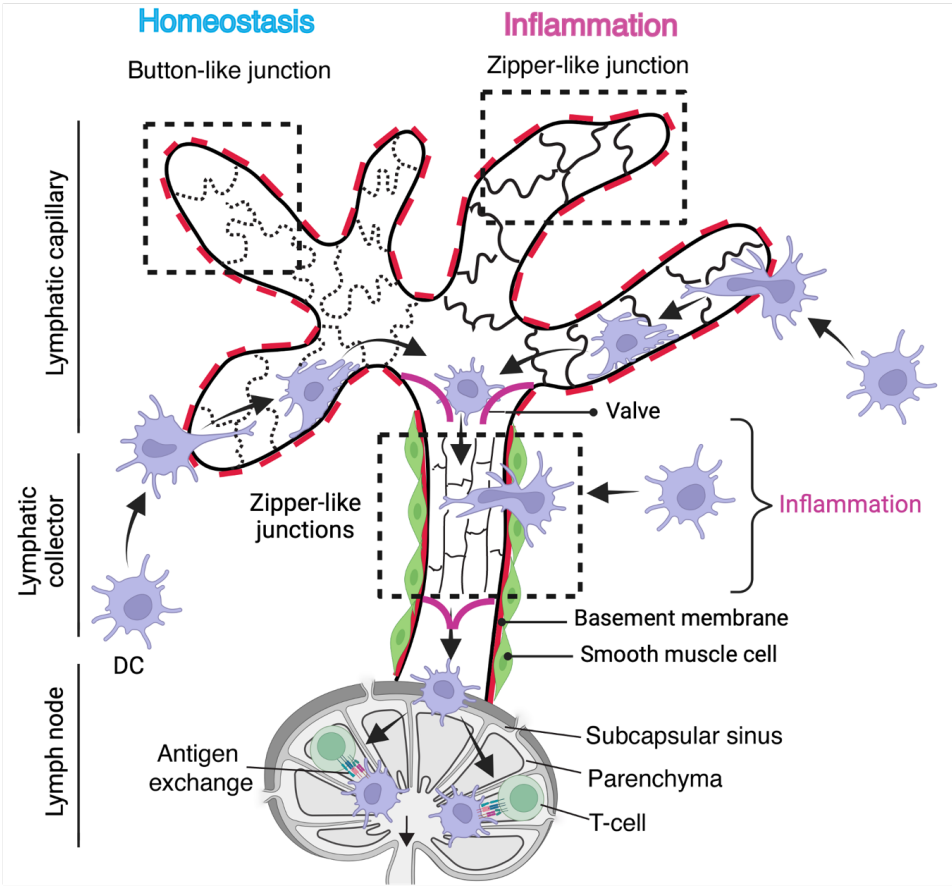


Figure 9: Dendritic cell transmigration into the lymphatic vessel. The schematic shows a simplified diagram of a lymphatic vessels consisting of lymphatic capillaries and merging into the lymphatic collector vessel and ultimately connected to the draining lymph node. In homeostasis, the junctions between the lymphatic endothelial cells (LECs) represent a button-like arrangement (Baluk et al., 2007). However, upon inflammation the LECs display zipper-like junctions, akin to the LECs of the collecting vessel (Yao et al., 2012). Most of the dendritic cell (DC) transmigration occurs at the level of the lymphatic capillaries, which have a discontinuous basement membrane (discontinuous red line) however, especially upon inflammation, DCs can also enter through the collecting vessels, which have a continuous basement membrane (red continuous line) (Arasa et al., 2021). After entering the lymphatic vessel lumen, the antigen-presenting DCs are finally homed to the lymph node where they present antigens to the T-cells (Steinman et al., 1997; Banchereau & Steinman, 1998). This causes their activation and the initiation of the adaptive immune response (Forester et al., 2012). This schematic has been modified from (Jackson, 2019) and published thesis by Ida Hilska (2024) from Vaahtomeri lab, titled "The guidance of dendritic cells to the transmigration sites on lymphatic endothelium", CAN PRO, University of Helsinki.
https://helda.helsinki.fi/server/api/core/bitstreams/125b7dcb-81d3-4559-9489-1b07e36f7453/content. The schematic was created using BioRender.com.

Upon reaching the basement membrane, the DCs are guided, through pre-existing pores, to the endothelial barrier under the influence of chemokine CCL21 (Pflicke & Sixt, 2009). Intriguingly, these pores enlarge upon DC movement, allowing their easy passage (Pflicke & Sixt, 2009). Upon reaching the lymphatic endothelia, the DCs need to breach the LEC barrier to gain access into the lymphatic vessel lumen. A previous study showed that upon forming a contact with the lymphatic vessel surface, the DC applies a mechanical push on the LECs. Moreover, the DC extends a "foot-like" projection, which probes the LEC surface for the site of transmigration. This projection first forces its way through the transmigration site and then the rest of the DC body follows (Vaahtomeri et al., 2017).

In addition to the approach of DC, chemokine CCL21 also plays a role in the transmigration of DCs. CCL21 is stored in vesicles after its production and packaging in the trans-Golgi network (Weber et al., 2013). This stored chemokine is available for an induced secretion for example, upon DC contact with the LECs or upon other stimuli, for example by encountering inflammatory cytokines (Johnson and Jackson, 2010; Vaahtomeri et al., 2017; Weber et al., 2013). A previous study showed that DC-LEC contact triggered an on-demand release of chemokine CCL21 (Vaahtomeri et al., 2017). The triggered release of chemokine CCL21 and the mechanical force exerted by the DCs, facilitates the breaching of the LEC barrier, therefore enabling their entry into the lymphatic capillary lumen.

Intriguingly, previous studies have also shown that many more DCs can enter the lymphatic vessel lumen using the same path, which was created by the first transmigrating cell (MartIn-Fontecha et al., 2003; Sen et al., 2010; Tal et al., 2011). However, why this occurs, is not clear. There is therefore something special about these transmigration sites, as compared to the rest of LEC surface, which makes them the "preferential" site of DC entry into the lymphatic vessel. It could be envisioned that the first cell leaves a trail of cues, which can be sensed by other cells that follow (Schumann et al., 2010; Vaahtomeri et al., 2017). Nonetheless, how DCs find the transmigration sites in the first place was not known and is explored in this thesis.

5.2.1 Role of adhesion for dendritic cell entry into the lymphatic vessels

Adhesion molecules are well known to facilitate leukocyte diapedesis across the blood endothelia (as explained in section 4.2). The first studies done in lymphatics in this context showed that adhesive interactions, between the LEC surface ICAM-1 and DC surface LFA-1, are necessary for DC transmigration into the lymphatics and their eventual homing to the lymph nodes (Ma et al., 1994; Xu et al., 2001). However, a later study showed that in homeostasis, integrin knockout DCs normally found their way to the lymphatics and transmigrated into them without a problem (Lammermann et al.,

2008). Therefore, it was thought that probably the integrins did not play a role in DC migration. Nonetheless, Johnson et al., have shown that upon inflammation the surface expression of ICAM-1 and VCAM-1, is upregulated on the LEC surface (Johnson et al., 2006). Moreover, upon inflammation, the interaction between the LEC surface ICAM-1 and VCAM-1 with the DC surface LFA-1 and VLA-4 becomes relevant (Johnson et al., 2006; Teoh et al., 2009). Further evidence to support this showed that blocking these interactions inhibited the transmigration of DCs into the lymphatic vessels (Teijeira, 2013; Arasa et al., 2021). Therefore, upon inflammation, integrin mediated binding of leukocytes with the LEC surface is required for their diapedesis into the lymphatic vessel lumen. As speculated in (Teijeira, 2013), this could be necessary as upon inflammation the lymphatic endothelial cell junctions of the lymphatic capillaries change from a "button" confirmation to "zipper-like" form, which reduces their permeability (Yao et al., 2012).

Additionally, both LECs and Langerhans DCs express L1CAM which, via homophilic interaction or by interacting with surface integrins, can provide adhesion between the LEC surface and the interacting DC (Pancook et al., 1997; Maddaluno et al., 2009). L1CAM has also been implicated in supporting the transmigration of DCs across the lymphatic endothelia. However, the molecular mechanisms which control this interaction are not yet known (Maddaluno et al., 2009).

5.3 Mechanism of lymphatic endothelial cell junction opening

Only a limited understanding currently exists of how LEC junctions are modulated during the transmigration events and the molecular mechanisms which regulate their opening and resealing, also remain to be understood.

However, as explained in sections above, the LEC-LEC junctions employ several adhesion molecules to ensure their proper sealing and control their permeability. It could be envisioned that the opening of the LEC junctions requires endocytosis/rearrangement of VE-cadherin and other LEC junction adhesion proteins (see section 5.1.2 for LEC junction adhesion molecules). This could for example occur via the disruption of junctional VE-cadherin, as explained in the opening of BEC junctions (see section 4.5).

A previous study by Sixt lab (IST, Austria) showed that, DC contact with the LEC monolayer induces calcium signaling in the LECs, by increasing cytosolic Ca^{+2} ions and that triggers the release of chemokine CCL21 (Vaahtomeri et al., 2017). The increase in intracellular Ca^{+2}, could also in principle lead to the activation of myosin light chain kinase and the ROCK mediated downstream rearrangement of actin cytoskeleton, as explained for the blood endothelia (Carbajal et al., 2000; Muller,

2003) (see section 4.5). However, actual experimental data to support that in LECs does not exist.

Furthermore, it was shown that the binding of DC surface semaphorin 3A (sema3A) with LEC surface receptor complex, plexin-1/neuropilin-1, is essential for DC transmigration into the lymphatic vessels. Ablation of this interaction inhibited DC transmigration (Takamatsu et al., 2010). It was shown that the sema3A/plexin-1/neuropilin-1 axis controls actomyosin contractions and the rearrangement of DC cytoskeleton, via the Rho kinase ROCK mediated activation of myosin II, which is necessary for the opening of the transmigration site (Takamatsu et al., 2010).

Numerous other pathways can also induce the activation of the cell contractility machinery and thereby lead to the opening of cell junctions. For example, sphingoshine-1-phosphate (S1P) mediated Cdc42/Rac/Rho signaling, can induce the rearrangement of LEC cytoskeleton and also supports DC mobility. The S1P receptor-1 (S1PR1) is expressed by DCs and the LECs (Karuppuchamy et al., 2017). Blocking S1PR1 receptor in DCs with a chemical inhibitor, severely obstructed their movement in vitro and *in vivo* (Czeloth et al., 2005). Based on this knowledge, it is possible that several pathways work together to facilitate the intravasation of DCs into the lymphatic vessel lumen. However, further experimentation is required to dissect the exact mechanisms of LEC junction opening.

6. Spatiotemporal control of guidance cues

The migration of leukocytes from the blood to the tissues and their intravasation into the lymphatics is a carefully orchestrated process where the leukocytes migration is directed at the correct place and time. Leukocyte diapedesis across the blood endothelia is a well-studied process. On the other hand, very little is known about how the leukocytes are guided to the lymphatics and how they enter them. Interestingly, previous studies have indicated that neutrophils tend to migrate preferentially through tri-cellular junctions across the blood endothelia (Burns et al., 1997; Wang et al., 2006; Gorina et al., 2014). However, how they locate the BEC tri-cellular junctions in the first place, is not known.

Additionally, it was known that several leukocytes can adopt the same path for transmigration into the lymphatics (Sen et al., 2010; Tal et al., 2011) however, the identity of these sites was not described in the context of lymphatics. It is possible that the leukocytes arrive at the transmigration sites randomly. On the other hand, it is also possible that the transmigration sites are enriched with guidance cues which attract the migrating leukocytes to these sites and drive their transmigration. However, the identity and mechanism of such a guidance is not clear, in case of the lymphatics. Here in this section, I will explore what is already known about leukocyte guidance across the lymphatic endothelia and what gap in knowledge existed which led to the aims of my research.

6.1 Guidance of dendritic cells

Ex vivo studies done with tracheal DCs, isolated from rats, showed that in homeostasis, the immature DCs display a set of steady-state receptors including CCR1, CCR2, CCR3, CCR5, CCR6, CCR10 and a few other proteins. This makes them sensitive to the presence of chemokines CCL2, CCL3 CCL5, CCL7, CCL13, CCL20 and eotaxins (Yanagihara et al., 1998; Stumbles et al., 2001; Feng et al., 2021). Also, see Figure 12 in the results section (section 8.3) of this thesis for all possible chemokine and chemokine receptors pairs in LECs and DCs. It can be envisioned that the migration of immature DCs occurs by sensing these steady-state chemokines. Experimental proof of this was presented in another study, which reported that CCL3 (MIP-1α), CCL5 (RANTES) and CCL7 (MCP-3) can mediate the migration of immature DCs (Yanagihara et al., 1998). Apart from that, other chemokines which can drive the migration of DCs are CCL19 and CCL21. It has been shown that chemokine CCL21 is constitutively produced by the lymphatic endothelium and is secreted in the interstitial tissue space and in lymph nodes (Johnson & Jackson, 2010; Ulvmar et al., 2014). DCs can detect CCL21 via their cognate receptor CCR7 (MartIn-Fontecha et al.,

2003). Although, CCR7 receptor is expressed upon maturation of DCs (Dieu et al., 1998; Sallusto et al., 1998), a small population of immature DCs, patrolling in the interstitial tissue space, can somehow increase their expression of CCR7 (Oppenheim et al., 2002; Alvarez, 2008). This causes their attraction and transmigration into the lymphatics (Ohl et al., 2004).

Previous studies have shown that the disruption of inter-membrane contacts between LCs, could result in the phenotypic maturation of LCs (Jakob & Udey, 1998; Riedl et al., 2000). Intriguingly, a study showed that homotypic interactions between DCs occur via E-cadherin mediated contacts and the disruption of these contacts can induce upregulation of CCR7 (Jiang et al., 2007). Although the DCs activated in this way also migrate towards the lymphatics, they have a different transcriptional profile, as compared to the DCs matured via antigen acquisition (Jiang et al., 2007). Apart from that another chemokine produced by the lymphatics is CXCL12, which is sensed by the patrolling DCs, via their CXCR4 receptor (Kabashima et al., 2007). CXCR4/CXCL12 signaling has also been reported to control the localization of DCs (Gallego et al., 2021). Obstructing this signaling axis, hindered the migration of cutaneous DCs (Kabashima et al., 2007). Therefore, in homeostasis, chemotactic guidance of leukocytes helps in the directed migration of leukocytes to the places where they are needed.

Intriguingly, upon antigen capture, the now mature DCs downregulate their steady state receptors, while at the same time they upregulate for example, CCR7 and CXCR4 (Dieu et al., 1998; Sallusto et al., 1998; Yanagihara et al., 1998). The CCR7 receptor recognizes both chemokines CCL21 and CCL19, which were reported to be essential in driving the migration of DCs in inflammation (Yanagihara et al., 1998; Forster et al., 1999). The chemokine CCL19 is primarily expressed by the fibroblastic reticular cells (FRC) of the lymph node (Luther et al., 2000). Whereas, the chemokine CCL21, is expressed by the endothelial cells of the lymphatic capillaries, Payer's patches and high endothelial venules and by the FRCs of the lymph node tissue (Gunn et al., 1998; Luther et al., 2000; Manzo et al., 2007; Weber et al., 2013; Ulvmar et al., 2014). In mice two different variants of CCL21 are present: CCL21-serine (produced by the lymph node and lymphatic capillary LECs) and CCL21-leucine (produced only by the lymphatic capillary LECs). Nonetheless, in humans only one type of CCL21 is known to exist (Vassileva et al., 1999). Previous studies have indicated that the production of chemokine CCL21 by the LECs is tripled upon inflammation, which orchestrates the migration of antigen-presenting DCs towards the lymphatics and assists their migration into the lymphatic vessel lumen (Johnson & Jackson, 2010; Teijeira et al., 2012).

Upon secretion, the chemokine CCL21 diffuses into the surrounding tissue. The positively charged C-terminus of the chemokine CCL21 can bind collagen IV,

chondroitin sulphate and heparan sulphate, present on the cell surfaces and ECM (Hirose et al., 2001; B. G. Yang et al., 2007). The immobilization of chemokine CCL21, results in the creation of an interstitial gradient, with highest concentration at the lymphatic vessel surface and subsequently diminishing into the interstitial space (Weber et al., 2013). Previous reports have also implicated ACKR4 in generating the CCL21 gradient in steady-state skin (Bastow et al., 2021). Patrolling, DCs sense the interstitial gradient of CCL21 and this drives their approach to the lymphatic vessels (Weber et al., 2013; Vaahtomeri et al., 2021). Interestingly, DC contact with the immobilized CCL21 can cleave the body of CCL21 at the binding site (C-terminus), and thereby result in a gradient of soluble CCL21, in the surrounding tissue (Schumann et al., 2010). This soluble CCL21 (without C-terminus) resembles chemokine CCL19 and provides guidance to the nearby DCs via CCR7 signaling (Schumann et al., 2010).

It has been reported that the deletion of LEC heparan sulphate can diminish the homing of DCs to the lymph nodes (Bao et al., 2010). However, a later study reported that the deletion of LEC heparan sulfate neither distorted the interstitial gradient of CCL21 nor the directed migration of DCs towards the lymphatics (Vaahtomeri et al., 2021). Also the CCL21 on the lymphatic vessel was very modestly changed and the DCs homed to the lymph nodes normally (Vaahtomeri et al., 2021). Based on that, Vaahtomeri et al., proposed that the heparan sulfate is dispensable for the formation of functional gradient of CCL21. On the other hand, cell culture and *ex vivo* evidence exists, showing that inflamed LECs and lymphatic vessels upregulate their expression of CD-137, which helps in the presentation of chemokine CCL21 to the DCs (Teijeira et al., 2012). In short, chemokine CCL21 is a decisive guidance cue, which is required for the intravasation of DCs into the lymphatics.

Furthermore, a previous study indicated that in the dermis, CCL21 was enriched at the LEC junctions upon transmigration of DCs. Furthermore, cell culture experiments indicated that the exocytosis of CCL21 and the DC transmigration depended on the influx of Ca^{+2} ions (Vaahtomeri et al., 2017). This on-demand release of chemokine was distinct from the long-range gradient in the interstitial space (Weber et al., 2013). However, it was not known if the CCL21 contributes to the guidance of DCs to the transmigration sites, on the plane of LECs. This question led to the aims of my thesis, which are addressed in the results and discussion section below.

6.2 Intralymphatic guidance of dendritic cells

Once inside the lymphatic capillaries, the DCs migrate to the lymph nodes, via the collectors (Figure 9). Their migration is assisted by the flow of the lymph, which creates a gradient of CCL21 inside the lymphatic vessel lumen (Dixon et al., 2005; Russo et al., 2016). The LECs present in the sub-capsular sinus of the lymph node

express chemokine CCL21 (Ulvmar et al., 2014). However, at the same time the LECs present on the ceiling (top) of the of the sub-capsular sinus also express ACKR4 (or CCRL1) receptor. Whereas the LECs present on the floor (bottom) of the sub-capsular sinus do not express this receptor (Ulvmar et al., 2014). ACKR4 acts as a sink by binding chemokines CCL21 and CCL19. This creates a chemokine gradient inside the SCS of the lymph node, which attracts the DCs towards the floor of the SCS, where they transmigrate and eventually interact with the T-cells (Figure 9) (Ulvmar et al., 2014). Specifically, the gradient of CCL21 was shown to be essential in driving the migration of DCs from the sub-capsular sinus (Ulvmar et al., 2014).

Additionally, *ex vivo* and cell culture evidence showed that in the lymph nodes, CCL21 can be immobilized on the FRCs, via binding to heparan sulfate, in the T cell area. This immobilized CCL21 mediates the binding of DCs, via the activation of β2 integrins (Schumann et al., 2010). A previous study reported that DCs lacking β2 integrin did not home to the interfollicular area, in the *ex vivo* section of lymph node (Schumann et al., 2010). Additionally, DCs lacking the CCR7 receptor failed to display directed migration in tissue cryosection and in cell culture, despite successful binding (Schumann et al., 2010; Ulvmar et al., 2014). Additionally, it was also reported that DCs failed to transmigrate into the parenchyma of the ACKR4-deficient lymph nodes, possibly due to the disruption of the CCL21 gradient in the lymph node (Ulvmar et al., 2014). This shows that sensing of chemokine CCL21 gradient is required for the guided migration of DCs (Braun et al., 2011).

In short, directed migration of DCs to the lymphatics is tightly regulated. Several factors and signaling pathways determine the extent of DC migration. Nonetheless, the one most crucial pathway in DC guidance to the lymphatics is the CCR7/CCL21 signaling route. Understanding the molecular mechanisms which control the secretion of chemokine CCL21 can therefore provide valuable knowledge and provide an opportunity to control the extent of DC migration into the lymphatic system. Additionally, since DC transmigration across the lymphatic endothelium represents a bottleneck in the process of their homing to the lymphatic vessels and the lymph nodes, knowledge about the identity of these transmigration sites in LECs could provide target areas to control the ingress of DCs into the lymphatic vessels and their homing to the lymph nodes. This could provide an opportunity to affect the extent of T-cells activation and control the adaptive immune response.

Aims of the study

Directed migration of cells plays a crucial role in the normal functioning of various biological processes, including development, leukocyte circulation, wound healing and immune surveillance. It is known that the homing of antigen presenting DCs to the lymph nodes, is required for the activation of T-cells, which further initiates the adaptive immune response (Forster et al., 2012). To reach the lymph nodes, the antigen-presenting DCs, follow the gradient of chemokine CCL21 in the interstitial space, which enables their entry into the lymphatic vessel lumen (Ohl et al., 2004; Tal et al., 2011; Weber et al., 2013; Vaahtomeri et al., 2017), and later in the lymph node (Ulvmar et al., 2014). To enter the lymphatic vessel lumen, the DCs transmigrate across the lymphatic endothelial cell (LEC) barrier (Forster et al., 2012). The LEC barrier therefore represents a bottleneck, which, by controlling the extent of DC intravasation into the lymphatic vessels, determines the extent of the adaptive immune response. It was known, that upon DC-LEC contact, the DCs trigger an on-demand secretion of CCL21, which promotes their migration across the LEC barrier (Vaahtomeri et al., 2017).

However, neither the identity of LEC chemokine storage compartments nor the processes which control chemokine secretion in LECs were known. Additionally, it was not known if the secretion of chemokine CCL21 plays a role in the guidance of DCs on the plane of LECs, to the transmigration sites, or if it is a random process? Moreover, it was not known, if LECs exhibit preferential sites of leukocyte transmigration, akin to the extravasation of leukocytes across the blood vessels (Burns et al., 1997; Wang et al., 2006; Sumagin & Sarelius, 2010; Gorina et al., 2014). This knowledge could provide the tools to control the transmigration of DCs into the lymphatics and the related activation of T-lymphocytes and therefore allow manipulation of the adaptive immune response. Based on this the specific aims of this study are:

(i) to identify the sites of DCs transmigration across the lymphatic endothelia
(ii) to identify the LEC chemokine CCL21 storage and secretory compartments
(iii) to identify the control mechanisms of chemokine presentation in LECs.

Material and methods

7.1 DNA constructs

To characterize the identity of chemokine CCL21 containing vesicles in LECs, I prepared a mini-library of EGFP-tagged RAB-GTPases, which are known markers of vesicle types including secretory vesicles and endosomes in cells. To do that end, numerous sub-cloning strategies were employed. These methods included restriction digestion-based recombination, Gateway, and Gibson (Gibson et al., 2009) sub-cloning techniques (Table 2). Apart from the already published constructs: pLenti6.3-CCL21ΔC-mCherry, pLenti6.3-CCL21-mCherry, and pLenti6.3-EB3-mCherry (Vaahtomeri et al., 2017; Kopf et al., 2020), the novel constructs generated for this study (I), are listed below (Table 2).

Table 2: Novel DNA constructs synthesized for this thesis

Construct	Source of cDNA	Cloning method
pENTR221-EGFP-RAB 1A, 2A, 3D, 5C,6A, 7A, 10, 13, 27A, 35 and 37	Orfeome or MGC libraries at the Gene Biology Unit (GBU), University of Helsinki, Finland	PCR amplification followed by restriction digestion or Gibson sub-cloning methods
pENTR221-EGFP-RAB 8A, 8A T22N, 11A	Johan Peränen (Hattula et al., 2002)	PCR amplification/Att-site insertion and Gateway BP clonase (Invitrogen, 11789020) mediated conversion
pENTR221-mCherry-RAB27A	pENTR221-EcoRI-RAB2A-BamHI (Vaahtomeri lab), pENTR221-CCL21-mCherry (Vaahtomeri et al., 2017) and pENTR221-EGFP-RAB27A (from above)	PCR amplification followed by restriction digestion and Gibson sub-cloning methods
Signal peptide (SP) of CCL21-mCherry	pENTR-D-TOPO-CCL21-mCherry (Vaahtomeri et al., 2017)	Mutagenesis PCR

After the sub-cloning steps, the above-mentioned constructs were introduced into chemically competent DH5α derived *Escherichia coli* cells (NEB 5-alpha, New England Biolabs, C2987H). Next, 30-70 µl of the transformed bacterial culture was

spread on growth plate (with appropriate antibiotics), and incubated overight at 37 °C. Next day, numerous isolated colonies were randomly picked, suspended in 50 µl of water and colony PCR was done to confirm the identity of the correct clones. The identified colonies were then used to seed and grow a mini-prep culture overnight. After propagation of the culture, the DNA was extracted using DNA isolation kit (Machery Nagel, 740588.50). Next, the integrity of the extracted DNA was confirmed using restriction digestion/gel electrophoresis and sebsequently, with sanger sequencing (Eurofins Genomics, Germany). Next, Gateway LR conversion kit (Invitrogen, 11791-020) was employed to convert the pENTR221-cDNA constructs into pLenti 6.3-cDNA constructs, using manufactirer's instructions. Later, One Shot Stabl3 (Thermo Scientific, C737303), chemically competent *Escherichia coli* cells were transfomed with the prepared pLenti constructs. Then mini-prep cultures were seeded and grown overnight after which the DNA was extracted, using DNA isolation kit (Machery Nagel, 740414.10), and stored for future experimentation.

7.2 Lentivirus production

Lentivirus production was carried out in LentiX-293 cell line (Chemicon, Darmstadt, Germany) using the protocol available at the Functional Genomics Unit (FuGU), University of Helsinki (https://www.helsinki.fi/en/infrastructures/genome-editing-function-and-stem-cell-platform/infrastructures/helsinki-virus-core-units/biomedicum-virus-core-unit/resources).

First, HEK293FT cells were grown to 80% confluent monolayer, on a 10 cm cell culture dish. The cells were cultured in complete high glucose Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 1% L-glutamine (Corning, 25-005-CI), 10% Fetal Bovine Serum (FBS) (Biowest, S181B-500), and 1% mix of Penicillin and Streptomycin antibiotics (Lonza, DE17-603E). On the day of lentivirus production, for each 10cm cultured HEK293 cell plate, two Eppendorf tubes were separately prepared: Tube 1 contained 500 µl Optimem media (Gibco, 11058-021) + 30 µl of Lipofectamine 2000 (Invitrogen, 11668-027) and, Tube 2 contained; a) 5.4 µg of the desired pLenti6.3 construct, b) 4 µg of pΔ8.9 (packaging plasmid), and c) 2.7 µg of pCMV-VSV-G (viral coat plasmid). The contents of each tube were gently mixed and incubated for 5 min at room temperature. After that, the contents of both tubes were mixed gently and incubated together for 20 min. In the meantime, the media from the cultured cells was removed, the cells were washed once with phosphate buffered saline (PBS), and 5 ml of Optimem media was added and the cells were returned to the incubator. Next, the transfection media was added dropwise on top of the cultured cells and the cells were incubated for 4-6h at 37 °C. After the incubation, the media was carefully removed and fresh 10 ml complete DMEM was added, and the cells were grown overnight. Next day, the culture media was removed and replaced with 8 ml of complete DMEM (without antibiotics), and the cells were cultured further for two overnights, without changing

the media. The supernatant was then carefully removed and filtered using 45 µm syringe filter (Sartorius 16537). The filtered media containing the lentivirus, was then aliquoted in cryo-tubes (Greiner 121263), snap frozen in liquid nitrogen and stored at -80 °C for future experimentation.

7.3 Mouse experiments

To study the colocalization of endogenous RAB6A with CCL21 in the lymphatic collecting vessels and capillaries and to observe the migration of LPS-activated dendritic cells (DCs) into the lymphatic vessels, C57BL/6JRcc mice were produced at Laboratory Animal Center, University of Helsinki. Tissue samples were collected at ages 8-12 weeks. The following animal handling licenses were employed: KEK21-031 (Laboratory Animal Center, University of Helsinki, Finland), ESAVI/30523/2019, ESAVI/40857/2022, and ESAVI/27566/2023 (project authorization board in Finland).

7.4 Lymphatic endothelial cell culture

Primary human dermal LECs (Promocell, C-12216) were cultured in MV2 media (Promocell, C-22022), added with all the supplements provided in the media supplement pack (Promocell, C-39221). Depending on the experiment, the cells were cultured either on gelatin coated 10-well slide (Greiner, 543078), 18-well slide (Ibidi, 81817), 13 mm cover slips (No.1), or 48-well cell culture plates (Thermo Scientific, 150687).

7.5 Preparation of bone marrow and dendritic cell culture

Dendritic cells were derived from the bone marrow extracted from the femur and tibia bones of control or CCR7$^{-/-}$ mice (C57BL6) (Forster et al., 1999), using the protocol described in (Vaahtomeri et al., 2017). The bones were a gift from Dr. Michael Sixt's laboratory (IST, Austria). After extraction, the bone marrow was cultured in R10 media (RPMI-1640 media (Corning, 15-040 or EuroLone, ECB9006L), containing 10% supernatant from cultured GM-CSF hybridoma cells (a gift from Dr. Michael Sixt's laboratory (IST, Austria), 1% L-glutamine (Corning, 25-005-CI), and 1% mix of Penicillin and Streptomycin antibiotics (Lonza, DE17-603E)). The media was replaced on day 3 and 6. On day 8, the media was collected and centrifuged (180 RCF) to pellet the DCs. The cells were resuspended in freezing media containing 90% FCS and 10% DMSO and stored in liquid nitrogen storage.

For DC culture and experimentation, the frozen ampule was thawed and diluted in R10 media (explained above). The DCs were pelleted by centrifugation (180 RCF) and resuspended in fresh R10 media containing 200 ng/ml of *Escherichia coli* O26:B6

derived LPS - lipopolysaccharide) (Sigma L2654). The culture was incubated at 37 °C for 21 h, after which the cells were pelleted again, resuspended in R10 media containing NucBlue live cell stain (Invitrogen, R37605) or Deep Red Cell Tracker (Invitrogen, C34565). The stained DCs were centrifuged (180 RCF), resuspended in MV2 media and loaded on top of cultured LECs or on mouse ear dermis tissue, maintained in R10 media.

7.6 Lentiviral transduction of cultured LECs

Cultured primary human dermal endothelial cells were incubated for 5-6h with transduction media consisting of lentivirus, 8 μg/ml polybrene (Sigma, H9268) and MV2 media. After the incubation, the cells were washed with phosphate buffered saline (PBS) and the transduction mix was replaced with MV2 media containing antibiotics: gentamicin and amphotericin (GA) (Lonza, cc-4083).

7.7 siRNA mediated knockdown

Target genes, in cultured LECs, were inhibited using siRNA mediated knock down. To that end, LECs were seeded on 18-well slide (Ibidi, 81817), or 48-well cell culture plates (Thermo Scientific, 150687) and cultured overnight in full MV2 media (Promocell C-22221), including all the supplements contained in the MV2 media supplement pack (Promocell, C-39221). The siRNA oligos were diluted to a stock concentration of 4 μM, in RNA dilution buffer (Dharmacon Horizon, catalog # B-002000-UB-100), according to the manufacturer's instructions.

Table 3: Preparation of siRNA transfection mixture

Material	18 well slide (Ibidi, 81817) (final volume 200 ml/well)	48 well plate (Thermo Scientific, 150687) (final volume 300 ml/well)
Transfection MV2/well	150 ml	225 ml
Diluted siRNA		
4 mM siRNA oligo	1 ml/reaction well (final concentration 20nM)	1.5 ml/reaction well (final concentration 20nM)
Opti-mem media	24 ml/reaction well	36 ml/reaction well
RNA-iMAX master mix		
RNA-iMAX reagent	0.6 ml/reaction well	0.9 ml/reaction well
Opti-mem media	24.4 ml/reaction well	36.6 ml/reaction well

On day 2, after starting the culture (approx. 16h-18h), the siRNA oligos were diluted separately for each sample well of the cell culture slide/dish. The siRNA oligos were diluted in reduced serum Opti-mem (Gibco, 11058-021) to a volume of 25 μl or 37.5

μl, depending on the culture well format (Table. 3). The final concentration of siRNA in each cell culture well was 20 nM (unless otherwise stated). Simultaneously, an appropriate volume of master mix was prepared according to the total number of sample wells (Table. 3). For example, for 10 wells of an 18-well slide (Ibidi, 81817), 6 μl of RNA-iMAX and 244 μl of Opti-mem reduced serum media were combined (see Table 3 for appropriate volumes for 48-well dish). The transfection mix and diluted siRNA were incubated separately for 5 minutes. After that, equal volume of RNA-iMAX master mix was added to each tube containing the diluted siRNA. This transfection mix was them gently mixed by flicking the tube manually and was incubated for 20 minutes at room temperature.

During the incubation, the MV2 media from the cultured LECs was removed, the cells were washed once with transfection MV2 (MV2 media including all the supplements from the supplement pack (Promocell, C-39221) but excluding fetal calf serum (FCS)). Finally appropriate volume (150 μl or 225 μl) (see Table 3) of transfection MV2 media was added to each well of the cell culture slide/dish and the cells were returned to 37°C incubator. After the 20-minute incubation of the transfection mix, it was added dropwise to each well of the cultured LECs and the media was mixed by pipetting up and down. The cells were incubated for 24 h at 37 °C, after which the media was replaced with normal MV2 (with all supplements and FCS). The siRNA oligos used in this study for silencing of genes are listed in (Table 4). For subsequent lentiviral transduction, the cells were allowed to grow for 2h in full MV2 media. After that, normal lentiviral transduction protocol (mentioned above), was employed.

Table 4: siRNA oligos used in this thesis

siRNA	Ref. No.	Source
siRAB6 pool	L-008975-00- 0005	Dharmacon/Horizon
siERC1 pool	LQ-010942-00-0002	Dharmacon/Horizon
siCTRL pool	D-001810-10-05	Dharmacon/Horizon
siCTRL-01	D-001810-01-05	Dharmacon/Horizon
siCTRL-02	D- 001810-02-05	Dharmacon/Horizon
siERC1-05	J- 010942-05	Dharmacon/Horizon
siERC1-06	J- 010942-06	Dharmacon/Horizon
siERC1-07	J- 010942-07	Dharmacon/Horizon
siERC1-08	J- 010942-08	Dharmacon/Horizon
siCCL21-05	J- 007833-05	Dharmacon/Horizon
siCCL21-06	J- 007833-06	Dharmacon/Horizon
siCCL21-07	J- 007833-07	Dharmacon/Horizon
siCCL21-08	J- 007833-08	Dharmacon/Horizon

7.8 Quantitative PCR analysis

The efficiency of siRNA mediated knock-down of targets was verified using quantitative PCR. For this, cultured primary LEC monolayers were transfected with siRNA oligos listed in (Table 5). The monolayer was then harvested by treatment with Trypsin-EDTA (Gibco, 25200056). The collected cells were pelleted by centrifugation (210 RCF) and kept on ice (4 °C) shortly, until subjected to RNA extraction or, stored in -80 °C storage for processing later. Next, for RNA extraction, the cell pellets were resuspended in lysis buffer included in the RNA isolation kit (Qiagen, 74104) and RNA was isolated according to the manufacturer's protocol. Next, NanoDrop™ spectrophotometer (NanoDrop™-ND-100) was used to measure the concentration of the extracted RNA. After that, the RNA was converted to cDNA using manufacturer's instructions included in the conversion kit (Applied Biosystems, Cat: 4368814). Next, qPCR reactions were set-up to a final volume of 20 µl in a skirted plate (4titude, 4ti-0740). The constituents of the reaction mix included: 4 µl cDNA and 10 µl of SYBR green mix (Merck, 4913850001) and 10 µM final concentration of primer pair mix (Table 5). The primers were designed using Primer3 tool (available at https://primer3.ut.ee) and the designed oligos were obtained from Sigma-Aldrich/Merck, Germany.

After adding the reaction components together, they were mixed by pipetting up and down while keeping the plate on ice. Next, the plates were sealed and analyzed via BioRad, C-1000 touch, CFx96 real-time qPCR thermocycler. The obtained Cq values were then exported to Microsoft Excel and the average of technical duplicates were calculated. These values were then used to calculate the ΔCt values by subtracting the Cq of knock down samples from the control samples. Next, the obtained ΔCt values were subtracted from ΔCt values similarly calculated for housekeeping gene (HPRT). The resulting ΔΔCt values were then used to obtain $2^{-\Delta\Delta Ct}$, which when multiplied with 100 gave the percentage expression of each mRNA. The results were plotted using Prism GraphPad (v.9.2.0).

Table 5: Primer oligos used for qPCR analysis in this thesis

Primer target	Sequence of primer pair	Source
HPRT	ATTATGGACAGGACTGAACGTCTTG, TGTAATCCAGCAGGTCAGCAAAG	Sigma-Aldrich/Merck
RAB6A	AGGAGGGAGAGAGGA AAGCC, CTTTCCATTCCCGGCAAAGC	Sigma-Aldrich/Merck
ELKS (ERC1)	ATGGCTGAAGAGAAGGGGAC, GTGTCAGTGTTGGTGGTGTC	Sigma-Aldrich/Merck
CCL21	TCCTTATCCTGGTTCTGGCC, GCAAGAACAGGATAGCTGGG	Sigma-Aldrich/Merck

7.9 Immunofluorescence staining of cells and mouse ear dermis

For immunofluorescence staining, cultured LECs were fixed with 4% paraformaldehyde (PFA) (Histolab, HL96753). Conversely, staining with anti-ELKS antibody required fixation with 1% PFA instead. After fixation the cells were permeabilized with 0.15% Triton x-100 (in PBS) for 15 min, washed with PBS and blocked with 3% bovine serum albumin (BSA) for 1 h. Later, the cells were incubated with primary antibodies overnight at 4 °C. Following day the cells were washed with PBS three times and incubated for 2h with secondary antobodies. After that the secondary antibody was removed, the cells were washed three times with PBS. Next the cells were incubated with DAPI (Tocris 5748) for 5 min, washed twice with PBS and stored at 4 °C in PBS containing 0.05% sodium azide.

Similarly, for staining of mouse ear tissue, the ear samples were first fixed with 1% PFA for 1h, washed and subsequently permeabilized with 0.3% Triton x-100 for 30 minutes. Next, the samples were blocked for 1h with donkey immunomix (DDIM) containing; 5% donkey serum (Biowest, S2170), 0.2% Bovine serum albumin (Biowest, P6154), 0.05% sodium azide (Sigma-Aldrich, S2002) and 0.3% Triton X-100 (Fisher Bioreagents, BP151) prepared in Dulbecco's PBS (Corning, 21-030-CVR). Then, the samples were incubated with primary antibodies for 2-3 days at 4 °C. After that, the ear samples were washed with permeabilization buffer and Dulbecco's PBS and incubated with secondary antibodies for 2 days at 4 °C. Finally, the samples were washed and stored in 0.05% sodium azide solution until imaged. The list of primary antibodies used is provided in (Table 6).

Table 6: List of primary antibodies used for staining cultured LECs and ear tissue

Antibody, clone	Use/dilution	Manufacturer, Ref. No.
rabbit anti-VE-cadherin, D87F2	IF cells/1:200	Cell signaling, 2500S
mouse anti-VE-cadherin	IF cells/1:2000	BD bioscience, 555661
mouse anti-VE-cadherin-Alexa Fluor 647 conjugated	Live imaging IF/1:1000	BD pharmingen, 561567
rat anti-VE-cadherin	IF tissue/1:100	Biolegend, 138003
rat anti-VE-cadherin	IF tissue/1:100	Invitrogen, 14-1441-81
rabbit anti-RAB6, D37C7	IF cells and tissue/1:100	Cell signaling, 9625S
rabbit anti-RAB7, D95F2 XP	IF cells/1:200	Cell signaling 9367TS
mouse anti-CCL2, clone 24822	IF cells/1:100	R&D Systems, MAB279
mouse anti-α-Tubulin	IF cells/1:200	Santa-Cruz biotechnology, sc-32293
mouse anti-ELKS (E-1)	IF cells/1:200	Santa-Cruz biotechnology, sc-365715
goat anti-CCL21/6Ckine biotinylated antibody	IF tissue/1:100	R&D Systems, BAF457
rat anti-LYVE1	IF tissue/1:300	R&D Systems, MAB2125

rabbit anti-LYVE1	IF tissue/1:1000	Dr. Kari Alitalo lab (Karkkainen et al., 2004)
rat CD31-FITC antibody, 390	IF tissue/1:200	Thermo Scientific, 11-0311-82
mouse anti-CXCL12, clone 79018	IF cells/1:50	R&D Systems, MAB350 sp

7.10 Microscopy

High resolution imaging of fixed and live imaging of cell and tissues was carried out using the microscopes listed in (Table 7).

Table 7: Microscopes used for imaging of fixed and live cells and mouse ear tissue

Microscope	Objective lense	Detector/camera	Details and use
Andor dragonfly spinning disc	Plan Apo λ 20X dry (NA 0.75), plan Apo VC 60X water (NA 1.2), SR Apo TIRF 100X oil (NA 1.49)	Andor Zyla 4.2 sCMOS, Andor Sona sCMOS, Andor iXon 888 U3 EMCCD	Lumencor Sola light engine, 5 laser sources (404/488/561/642/730 nm) and Fusion 2.0 software. Used for imaging of fixed samples and for live imaging of cultured cells.
Zeiss LSM 880	Plan-APOCHROMAT 63X oil (NA 1.4)	PMT, GaAsP, Airyscan, PicoQuant FLIM	405/561 nm diode laser, 458/488/514 nm Argon laser, 633 Helium-Neon laser, beam splitter for airyscan mode and Zeiss Zen 2 software. Used for imaging of fixed cells and mouse ear dermis.
Leica Stellaris 8 FALCON/DLS (DMI8)	HC PL APO CS2 63X oil (NA 1.4)	5 HyD dectectors (HyD S and HyD X)	405nm diode laser and supercontinum white-light laser, Acusto optical beam splitter, SuperZ-galvometric stage and Leica LAS X 4.6.0 software. Used for imaging of fixed cells and mouse ear dermis.
Nikon Ti-E widefield	Plan Apo λ 20X dry (NA 0.75), plan Apo VC 100X oil (NA 1.4)	Hamamatsu Orca Flash 4.0 V2 B&W camera	Lumencor Spectra X light engine, 6 solid state light sources, lamp source for phase contrast with DIC polarizer and NIS elements software. Used for live imaging of cultured cells.

Leica SP8	HC Fluotar L 25X dipping objective (NA 0.95)	3 PMT detectors	405/488/552/638 nm lasers, and LAS-X software. Used for live imaging of DC migration on mouse ear dermis explant.

For live microscopy application all microscopes used were equipped with full enclosure atmospheric chamber (37 °C, 90% humidity and 5% CO_2). Live imaging of EB3-mCherry comets was done by Dr. Kari Vaahtomeri and Maria Saario (Vaahtomeri lab), using Andor spinning disc microscope with 100X objective. Whereas live imaging of EGFP-RAB6A and CCL21ΔC-mCherry vesicles was done using Nikon Ti-E widefield microscope and the 100X objective. For live imaging of DC transmigration across the LEC monolayer, Nikon Ti-E widefield microscope with 20X objective was used. This experimentation was performed by Ida Hilska (Vaahtomeri lab). LEC cell junctions in all cases were stained with non-blocking anti VE-cadherin antibody conjugated with Alexa Fluor 647, apart from live monitoring of DC transmigration into the mouse ear dermis lymphatics. In this case the lymphatics were stained with CD31-FITC antibody and the DCs were stained with Deep Red Cell Tracker dye (Invitrogen, C34565). This experiment was done by Dr. Kari Vaahtomeri, using Leica SP8 microscope with 25X dipping objective and full enclosure atmospheric chamber (37 °C, 90% humidity and 5% CO_2).

7.11 Transmission electron tomography

Human primary dermal LECs were grown on 0.1% gelatin coated 13 mm cover-slips (No.1). The cells were then lentivirally transduced to express CCL21-mCherry, CCL21ΔC-mCherry, EGFP-RAB6A or EGFP-RAB27A. After 2 days post lentiviral transduction the cells were fixed, permeabilized, blocked and stained (see Table 8). For fixation, permeabilization, blocking, antibody staining, post-fixation, silver enhancement and gold toning, the protocol supplied by University of Helsinki's electron microscopy unit (EMBI) was used. After that the samples were submitted to the electron microscopy unit (EMBI), for osmium treatment, epon embedding and sectioning. Sections were cut using ultracut UCT7, Leica Biosystems and collected on single slot copper grids precoated with pioloform. The ultrastructure of LECs was visualized at the University of Helsinki's electron microscopy unit (EMBI), using the Jeol JEM-1400 electron microscope. The microscope was operated at 80 kV and the images were captured using Gatan Orius SC 1000B CCD camera.

Table 8: List of antibodies used for transmission electron tomography

Antibody	use	Manufacturer, Ref. No.
Primary antibodies		
rabbit anti-EGFP	IF	abcam, ab290

rabbit anti-RFP	IF	Rockland, 600-401-379
Secondary antibody		
anti-rabbit nano gold particles	IF	Nanoprobes, Stony Brook, NY

7.12 Analysis of live microscopy videos

Numerous experiments required the analysis of live videos: (i) The exocytosis of CCL21ΔC-mCherry vesicles was quantified by manually counting the diasppearance of CCL21ΔC-mCherry+ vesicles near the LEC junctions (stained with VE-cadherin) in ImageJ, Image analysis software (v. 2.0.0-rc-69/1.52n). (ii) The number of DC transmigration events across the cultured LEC monolayer, or the mouse ear tissue lymphatics, were quantified manually by identifying the diapedesis events in the captured videos. The experimentation and quantification were done by Ida Hilska and Dr. Kari Vaahtomeri. Videos were visualized using ImageJ (v. 2.0.0-rc-69/1.52n). (iii) The trafficking routes of EGFP-RAB6+ vesicles, were visualized by creating a maximum projection of the fast-live videos in ImageJ (v. 2.0.0-rc-69/1.52n). (iv) To analyze the route of EB3-mCherry+ comets in relation to the LEC borders, Maria Saario (Vaahtomeri lab) manually marked the LEC bicellular and multicellular junctions, using the drawing tool in ImageJ image analysis software. After that, circular ROIs (10 μm in diameter) were drawn at the multicellular junction or alternatively, in the middle of the bicellular junction. The comets disappearing inside the circular ROIs or continuing forward, were identified and manually marked using the multi-point tool in ImageJ Image analysis software (v. 2.0.0-rc-69/1.52n). GraphPad Prism (v.9.2.0) software was used to plot the obtained results.

7.13 Colocalization analysis

To study the colocalization between proteins (immunofluorescence staining or overexpression of fluorescent tagged proteins), a semi-automated approach was employed. To detect the localization of proteins, automatic spot detection tool in IMARIS image analysis software (v9.5.0 and v9.8.2, Bitplane, Oxford instruments) was employed. Manual refining of spots was then done for the accuracy of detection. In doing so, the spots detected in the Golgi area were excluded. At the same time, dim vesicles were manually marked (for example in EGFP-RAB8A and CCL21ΔC-mCherry). The colocalization between CCL21ΔC-mCherry or CCL21-mCherry and EGFP-RAB-GTPases, CCL2, RAB6, RAB7, LAMP1 or LAMP3 was calculated using automatic colocalization calculation tool in IMARIS. Additionally, the VE-cadherin stained LEC junctions were manually segmented using drawing tool and the bicellular and multicellular junctions were manually marked using the object tool. Next, distance transformation maps were created in IMARIS, followed by calculation of the distance of colocalized vesicles from the nearest multicellular junction or LEC borders.

Conversely, colocalization between endogenous CCL21 and RAB6, in the mouse ear tissue was quantified using ImageJ image analysis software (v. 2.0.0- rc-69/1.52n). The LEC junctions were marked by manual drawing using the freehand line tool or multipoint tool. The colocalized and non-colocalized CCL21 vesicles were marked using the multipoint tool, as separate sets. Next, the distance of these set of vesicles from the nearest multicellular junction (in case of lymphatic pre-collectors with continuous VE-cadherin staining) or the LEC borders (in case of lymphatic capillaries with discontinuous VE-cadherin staining), was calculated using scripts by Ved Sharma (accessed on 31.01.2024 at 1100 h):
URL: https:// github.com/ved-sharma/Shortest_distance_between_objects

To investigate the colocalization between CCL21ΔC-mCherry and endogenous RAB6 upon siControl or siELKS treatment of cultured LEC, IMARIS image analysis software (v9.8.2) was used to mark the LEC multicellular junctions using the spot tool. Next, distance transformation maps were created and saved as ".tif" files. Then, ImageJ (v. 2.0.0-rc-69/1.52n), was used to create masks of the whole cell body and the masks, representing radius of 5 µm, over the LEC multicellular junctions. Then, using the XOR tool in ImageJ, the overlayed masks were reduced, generating the pies representing the multicellular vertices or the rest of the cell body. Next based on DAPI and RAB6 perinuclear/Golgi signal, the nuclei and Golgi area were segmented using the polygon tool and excluded in the final quantification. Later, thresholding tool was used to separately segment RAB6+ and CCL21ΔC-mCherry+ areas and individual masks were created based on these areas. The RAB6 and CCL21ΔC-mCherry masks were then multiplied to generate a new mask showing the colocalized area. This mask was then overlayed with either the mask representing the multicellular junction "pies" or the "rest of the cell body" masks. The measured fluorescenec intensity was then plotted using Prism GraphPad (v.9.2.0) and the results were compared between different treatments.

7.14 ELKS intensity analysis

To quantify the ELKS intensity at LEC junctions, we employed ImageJ image analysis software (v. 2.0.0-rc-69/1.52n). Using the freehand line and overlay brush tools, the LEC multicellular and bicellular junctions were segmented, based on the VE-cadherin signal. The ends of the drawn lines connected one multicellular junction to the next. Then, the intensity of ELKS signal, under the drawn lines, was measured using the multiplot tool. The data obtained was imported to Microsoft excel and tabulated showing ELKS intensity as a function of distance from the nearest multicellular junction. This was finally plotted using Prism GraphPad (v.9.2.0). Similarly, the effect of PI3K inhibition on ELKS intensity was quantified using the same analysis protocol.

7.15 Analysis of CCL21ΔC-mCherry and CCL2 intensity

To measure the fluorescence intensity of CCL21ΔC-mCherry or CCL2, ImageJ (v. 2.0.0-rc-69/1.52n) image analysis software was used. Two types of analyses were done: (1) intensity measurement in the whole cell or, (2) specifically at the LEC multicellular junctions. Since, endogenous CCL2 stained all the cells of the monolayer, therefore its average fluorescence intensity was measured per pixel, in the case of whole cell analysis. Background fluorescence intensity was measred from samples treated similarly, but not incubated with primary antibody. Conversely, for measuring the intensity of CCL21ΔC-mCherry, the cells overexpressing it were first segmented using the polygon tool. Next, fluorescence intensity was measured in the segmented cells. Non-transduced cells were used to measure the background signal.

To measure the CCL21ΔC-mCherry and CCL2 intensity, within 5 μm of the LEC multicellular junctions, the multicellular vertices were first marked using the spot tool in IMARIS (v9.8.2). Next, distance transformation maps were created in IMARIS and the data was exported in the ".tif" format. Next, ImageJ was used to create masks to segment the cells (in case of CCL21ΔC-mCherry) and then later the muticellular junctions (for both CCL21ΔC-mCherry and CCL2). Finally, the obtained masks of the multicellular junctions were overlayed on the z-slice from the hyperstack which showed the maximum signal intensity of CCL21ΔC-mCherry or CCL2. The obtained intesity values were then plotted and compared between different treatments.

7.16 Flow cytometry

To compare the contractility machinery proteins of wild type versus CCR7 $^{-/-}$ DCs, the cultured cells were collected and pelleted from the suspension by Dr. Kari Vaahtomeri. These were then resuspended on ice in buffered R10 media supplemented with 4% PFA. Fixation was performed for 15 min on ice, after which the cells were washed and subsequently blocked in FACS buffer (2 mM EDTA in PBS) supplemented with 1% BSA and rat anti-mouse CD16/31 antibody (BD Pharmingen Cat #: 553142). After, that the cells were incubated with antibodies (Table 9) for 1h at room temperature. The cells were washed, pelleted, resuspended in FACS buffer and analyzed using Novocyte Quanteon Flow Cytometer and Novoexpress software. Controls included, unstained DCs and compensation beads (Biolegend, 424602), which were incubated with the same antibodies (Table 9).

Table 9: List of antibodies used for flow cytometry (FC)

Antibody, clone	Use (dilution)	Manufacturer, Ref. No.
rat anti-CD11b-PE, M1/70	IF/FC (1:400)	BioLegend, 101208
rat anti-CD11c-PE-Cy7, HL3	IF/FC (1:300)	BD Pharmingen, 121421
anti-CD86-FITC, GL1	IF/FC (1:300)	BD Pharmingen, 561962
rat anti-MHCII-eFluo450, MS/114.15.2	IF/FC (1:800)	eBioscience, 48-5321-82

rat anti-CD16/31	IF/FC (1:100)	BD Pharmingen, 553142

7.17 Western blot

To compare the marker proteins commonly associated with cell contractile machinery, of wild type versus CCR7 $^{-/-}$ DCs, western blotting technique was employed. To that end, the cultured cells were collected, pelleted and lysed using treatment with SDS boiling buffer. This was followed by further breaking the cells with using a 25-gauge hypodermic syringe needle. Next, the lysate was centrifuged (18000 RCF) for 10 min at 4 °C and the supernatant was collected. Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, 23227) was then employed to measure the concentration of proteins. Next, SDS-PAGE and wet blotting membrane transfer methods were applied. The obtained membranes were blocked and incubated with antibodies (Table 10). The signal was then read using Azure 500 imaging system (Azure biosystems) and AzureSpot Pro (Azure biosystems, 1.9.0.0406) software. The experimentation was performed by Ida Hilska.

Table 10: List of antibodies used for Western blotting (WB)

Antibody, clone	Use/dilution	Manufacturer, Ref. No.
Primary antibodies		
mouse anti-b-actin, AC-15	WB/1:2500	Sigma, A1978
rabbit anti-non-muscle heavy chain IIA, Poly 19098	WB/1:1000	Biolegend, 909801
mouse anti-HSC70, B6	WB/1:2000	Santa Cruz Biotechnology, sc-7298
Secondary antibodies		
donkey anti-mouse IRDye 680RD	WB/1:5000	LI-COR
donkey anti-rabbit IRDye 800CW	WB/1:5000	LI-COR

7.18 Secreted CCL21ΔC-mCherry in culture media

To measure the effect of inhibiting RAB8A on the amount of secreted CCL21ΔC-mCherry, LECs were co-transduced with lentivirus encoding CCL21ΔC-mCherry and either of EGFP (control) or EGFP-RAB8A-T22N, in a 1:1 ratio. After that, the virus was removed, cells were washed, and the culture was maintained by regularly replacing the culture media every day for 2 days. On day 3 (72 h post lentiviral transduction and 24h from the last media change), the media from the culture was collected, centrifuged (300 RCF) for 6 min, and aliquoted to a round bottom 96-well plate (Perkin Elmer, 6005270). The fluorescence intensity was measured at excitation 587 nm and emission 610nm, using EnSight multimode plate reader (Perkin Elmer), equipped with Kaleido software version 3.0. Background signal was measured from the media collected from the cells with no transduction. The results were exported to Microsoft

Excel and the background signal was reduced from the transduced samples. The results were finally compared between EGFP and EGFP-RAB8A-T22N treated sample and plotted using GraphPad Prism (v.9.2.0).

7.19 Bulk RNA sequencing of LECs (unpublished)

To analyze the bulk mRNA of primary human dermal LECs, the cells were cultured on 0.1% gelatin coated wells of a 96-well plate (Gibco, 25200056). The LECs were either transduced with lentivirus encoding mCherry (1 experiment) or left untreated (2 experiments). The cultures were maintained until the cells were confluent (3-days from the start of culture). The cells were then treated with trypsin-EDTA, pelleted and resuspended in 50 µl of RNA later solution (Invitrogen, 10564445). The samples were stored at -80 ˚C until analyzed using NextSeq 500 sequencer, using bulk mRNA sequencing 3'UTR RNAseq "Bulkseq" method, at Dr. Päivi Saavalainen's lab, Translational Immunology Research Program, University of Helsinki, Finland. The Bulkseq method used for this experiment is explained in (Lemmetyinen et al., 2023). The obtained raw FASTQ files were further processed in R/Bioconductor package software using Deseq2 package (Love et al., 2014), by the sequencing facility. The resulting TMM normalized RNA-seq data was then sorted in Microsoft Excel. A list of all detected RAB-GTPases and chemokines was created (Figures 10A and 11A, unpublished results in this thesis). The results represent three independent experiments comprising of 8 samples. The data was then compared from three experiments and the results were plotted using GraphPad Prism (v.9.2.0).

7.20 Re-analysis of mouse *in vivo* mesenteric and dermal LEC single-cell RNA-seq data (unpublished)

To ascertain the expression of commonly known human expressed RAB-GTPases and chemokines (Baggiolini, 2001; Tourniaire et al., 2013; Hughes & Nibbs, 2018), I used the Broad institute's single-cell mRNA sequencing database of adult mouse mesenteric LECs and the Mäkinen lab's single-cell mRNA sequencing database of dermal LECs. To that end, I uploaded the list of human RAB-GTPases and chemokines, to the online databases (see database details and links below). The data were automatically analyzed and plotted by the online database interface, using the bubble-plot creation tool. The online databases can be accessed at:

Mesenteric LECs (data ref: NCBI Gene Expression Omnibus: GSE156320) (Gonzalez-Loyola et al., 2021). Broad institute's single-cell mRNA sequencing database: https://singlecell.broadinstitute.org/single_cell/study/SCP1821/foxc2-controls-adult-lymphatic-endothelial-specialization-function-and-gut-lymphatic-barrier-preventing-multiorgan-failure?genes=Phldb2#study-download

Dermal LECs (Data ref: Gene Expression Omnibus, GSE201916) (Petkova et al., 2023). Dr. Taija Mäkinen lab's single-cell mRNA sequencing database: https://makinenlab.shinyapps.io/DermaLymphaticEndothelialCells/

The bubble plots (Figures 10B and 11B, this thesis) show the single-cell mRNA expression levels of RAB-GTPases and chemokines in the mesenteric LEC. The bubble plots (Figures 10C (modified from EV4B in I) and 11C, this thesis) show the dermal LEC single-cell mRNA data. These plots were made without scaling the gene expression (This was an option available in the database bubble plot creation tool). These results could be compared to our bulk mRNA-seq data for RAB-GTPases and chemokines (Figures 10A and 11A, this thesis).

7.21 Statistical analysis

To test the normality of data Shapiro-Wilk or Kolmogorov-Smirnov tests were used. For normally distributed data two-tailed Student's t-test was used (including Welch's correction). Conversely, Mann-Whitney U test was used for non-normally distributed data. Fisher's exact test, Chi-squared test was employed for categorical data and ordinary ANOVA test was used for the analysis of grouped data.

7.22 List of contributions

The following table summarizes the techniques I used for the experimentation and the quantification and analysis I performed in this study. The contributions of others involved in my thesis project are mentioned along with the methods above.

Table 11: Techniques learnt, and experiments performed by me in this thesis:

No.	Techniques and analysis	Figure in (I) or unpublished results (this thesis)
1	Live microscopy and quantification	Published article (I): Fig. 2B-C, 4A & D, 5C-D, 6A-B, EV1P, Movies EV7 & 9.
2	Immunofluorescence staining: cells and tissue	Published article (I): Fig. 2G-L, 2K-L, 3D-F, 4B-C, 5A, E, I, 6A-J, 7C, G, H, EV1A, EV2A-C, H-I, EV3A-B, EV4A, EV5A.
3	Spinning disc and confocal microscopy	Published article (I): Fig. 2D, 2G-L, 3D-F, 4B-C, 5A, E, I, 6C-J, 7C, G, H, EV2A-C, H-I, EV3A-B, EV4A, EV5 A, D, G.
4	Electron microscopy	Published article (I): Fig. 3A-C.
5	Colocalization and other image analysis	Published article (I): Fig. 2D-E, 2M-N, 3G-H, 4A, 5C-D, 6A, G-J, 7A-B, 7H-K, EV1P, EV2A-K, EV3A-E, EV4A.
6	Vesicle distance analysis	Published article (I): Fig. 2F, N-P, 5D, EV2G, K, EV3E.
7	Lentivirus production and transductions	Published article (I): Fig. 2A-B, D, G-H, 3A-F, 4A-B & D, 5E & I, 6A-F, 7A-K, EV2A-C, H-I, EV4A.
8	siRNA mediated knock-down and quantification	Published article (I): Fig. 5C-J, 6C-J, EV1M, EV5D-G.
9	qPCR and quantification	Published article (I): EV1M, EV5 E-F.

10	Genetic cloning	Published article (I): EGFP and EGFP-RAB-GTPases in Fig. 2D-F, 3A-C, & I, 4A, EV2A-C, EV3A-B, RAB8A T22N in Fig. 7C, G, H and sp-mCherry in EV4A.
11	Fluorescence intensity in media and analysis	Published article (I): Fig. 7B.
12	Bulk mRNA analysis	Unpublished results (this thesis): Fig. 10A and 11A.
13	In-silico RAB-GTPase and chemokine single-cell mRNA data analysis	Unpublished results (this thesis): Fig. 10B and 11B-C.
14	Figure and video preparation	Published article (I): Fig. 1C-D, 2A-P, 3A-E, 4A-E, 5A-J, 6A-K, 7A-M, EV1A-D, J, M-P, EV2A-I, EV3A-E, EVA-B, EV5A-G, Appendix S2A-F, S3A-B, S4A-C, Movies EV2-5 and EV7-13.

Results and Discussion

8.1 Dendritic cells preferentially transmigrate via the LEC multicellular junctions using the CCR7/CCL21 axis

To verify if preferential diapedesis sites exist for DC transmigration across the lymphatic endothelia, we loaded LPS activated bone marrow derived DCs on top of cultured primary human dermal LECs. Previous studies have reported that the chemokine CCL21 is needed to attract the DCs to the lymphatic endothelium and is required for their transmigration into the lymphatic vessel lumen (Tal et al., 2011; Weber et al., 2013; Vaahtomeri et al., 2017). However, upon cell culture, LECs down-regulate their production of CCL21 (Wick et al., 2007). For the expression and visualization of chemokine CCL21, we transduced the LECs with lentivirus encoding CCL21-mCherry (Vaahtomeri et al., 2017). The LEC junctions were visualized with non-blocking VE-cadherin antibody pre-coupled with a fluorophore. Using live microscopy, we observed that after finding an appropriate transmigration site, the DCs got arrested for 3-15 min and eventually transmigrated, across the LEC monolayer (Fig. 1A and Movie EV1 in I). Intriguingly, we found that about 84% of DC transmigration occurred at the LEC multicellular junctions and only a small minority at the bicellular junctions (Fig. 1B in I).

To recapitulate this finding in mouse ear pinna dermal tissue lymphatics, we loaded LPS activated and fluorescently labelled DCs on top of mouse ear dermal explant, as described in previous studies (Pflicke & Sixt, 2009; Weber et al., 2013; Russo et al., 2016; Vaahtomeri et al., 2017; Arasa et al., 2021). Wherein, the lymphatic endothelial cell junctions were labelled with anti-CD31-FITC antibody (PECAM1 staining). Using live imaging of the pre-collector vessels, which display continuous junctions, similar to cultured LECs, we observed that 48.5% of DCs transmigrated at the LEC multicellular junctions followed by 24.2% DCs, which transmigrated across the bi-cellular junctions, very close (≤5 μm) to the nearest multicellular junctions (Fig. 1C-D, Appendix Fig. S1A-C and Movies EV2-5 in I). These results showed for the first time the preferred transmigration of DCs across the LEC multicellular junctions, akin to those reported earlier in neutrophil diapedesis across the blood endothelia (Burns et al., 1997; Wang et al., 2006; Sumagin & Sarelius, 2010; Gorina et al., 2014) and the integration of multiciliary cells into the *Xenopus* lung epithelium, preferentially at the

multicellular junctions (Ventura et al., 2022). In the light of these evidence, our finding about the preferential transmigration of DCs occurring at the multicellular junctions, supports the findings in other cell types.

Nonetheless, what guides the DCs to the preferential transmigration sites, was not known. It was previously reported that mature DCs display CCR7 receptor on their surface (Dieu et al., 1998; Sallusto et al., 1998; Yanagihara et al., 1998), which allows them to detect the chemokine CCL21, expressed by the lymphatic vessels (Ohl et al., 2004). The CCR7/CCL21 binding is necessary for the attraction of DCs to the lymphatics. Based on this we asked the question, if DCs use CCL21 to find these preferential transmigration sites, on the LEC surface. To that end, we acquired bone marrow from $CCR7^{-/-}$ mice (a generous gift from Prof. Michael Sixt lab, IST Austria), which was differentiated to derive $CCR7^{-/-}$ DCs. Next, we, loaded wildtype and $CCR7^{-/-}$ DCs, on top of LEC monolayer overexpressing CCL21-mCherry, and observed DC migration patterns. We found that $CCR7^{-/-}$ DCs exhibited non-directional random movement over the LEC surface (Fig. 1E-F and Movie 6 in I). Conversely, the wildtype DCs, more directly located the multicellular junctions and arrested there. Only 35% of $CCR7^{-/-}$ DCs were able to find the LEC multicellular junctions as compared to 63% in case of wildtype DCs (Fig. 1G in I). Moreover, almost all of the arrested wildtype DCs, transmigrated across the LEC monolayer. However, the $CCR7^{-/-}$ DCs despite the arrest, failed to transmigrate.

It is still not clear what causes the arrest of some of the $CCR7^{-/-}$ DCs at the LEC multicellular junctions. It is possible that besides CCL21, LEC multicellular junctions are also decorated with other unique factors, which make their detection by DCs possible. Previous studies have shown that during transcellular transmigration across BECs, the transmigration pore is decorated with ICAM-rich membrane projections which facilitate the transmigration event (Arts et al., 2021). Another in vivo study showed that during paracellular transmigration of leukocytes across the blood endothelium, the multicellular junctions were enriched with ICAM-1 which marked them as preferential sites of transmigration (Sumagin & Sarelius, 2010). It can be envisioned that, LEC multicellular junctions might as well recruit ICAM or other adhesion molecules which might make them more susceptible to host a transmigration event (Johnson et al., 2006; Johnson & Jackson, 2010; Sumagin & Sarelius, 2010; Teijeira et al., 2013; Arasa et al., 2021). Data from our experiments showed that indeed DCs locate the preferential site of transmigration by sensing CCL21, via their CCR7 receptor. I envision that, the CCR7/CCL21 signaling together with other LEC multicellular junction-specific factors mark these sites as preferential sites of DC transmigration. Further experimentation is required to identify all the proteins distinctively present at the LEC multicellular junctions.

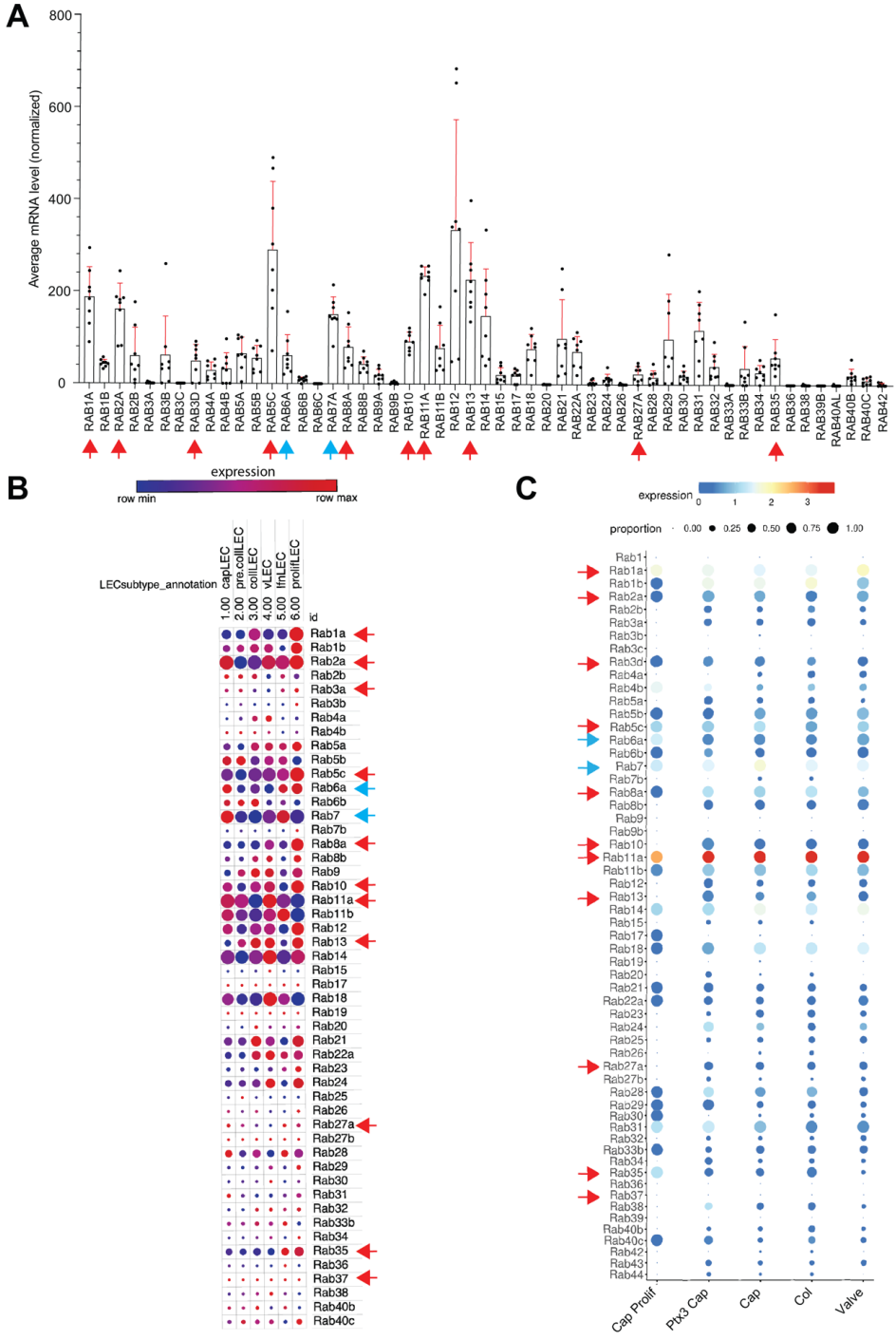
8.2 Chemokine CCL21 localizes in RAB6A+ and RAB3D/27A+ storage vesicles

8.2.1 Chemokine CCL21 localizes to two distinct vesicular populations in LECs

LECs constitutively produce chemokine CCL21, which is essential for DC transmigration into the lymphatics (Ohl et al., 2004). The secreted chemokine CCL21 forms a gradient in the surrounding tissue, which attracts the DCs, patrolling in the interstitial tissue space (Weber et al., 2013). Previous studies have reported that in LECs, the CCL21 localizes to the Golgi apparatus and the storage vesicles (Johnson & Jackson, 2010; Weber et al., 2013; Vaahtomeri et al., 2017). However, the identity of CCL21+ vesicles in LECs, was unknown. To visualize chemokine containing vesicles in LECs, I transduced primary human dermal LEC culture with lentivirus overexpressing fluorescently tagged CCL21, devoid of its C-terminal (CCL21ΔC-mCherry) (Figure EV1 C) (Vaahtomeri et al., 2017). This deletion of C-terminus was necessary, since full length CCL21-mCherry protein sticks to the LEC surface, via its positively charged C-terminus, by binding to the glycosaminoglycans and heparan sulphate present on the outer surface of LECs (Jorgensen et al., 2021). Moreover, the CCL21 also binds to the surface of the cell culture slide/coverslip, which creates an optical hue and makes observation difficult (Vaahtomeri et al., 2017). With the help of high-resolution spinning disc confocal microscopy, I observed the two distinct populations of CCL21ΔC-mCherry+ vesicles. These populations consisted of; (1) the higher intensity CCL21ΔC-mCherry vesicles, localizing around the nucleus (perinuclear pool) and, (2) the comparatively dim CCL21ΔC-mCherry vesicles, at the LEC multicellular junctions (peripheral pool) (Fig. 2A in I). This was an interesting finding, since we had observed that DCs preferentially transmigrated through the LEC multicellular junctions (Fig. 1A-B in I). This meant that the localization of the peripheral pool of chemokine CCL21 at the LEC multicellular junctions has a functional relevance, and possibly supports the migration of DCs, at these sites. Apart from that, in agreement with the previous reports we also confirmed the presence of CCL21 in the Golgi/trans-Golgi area (Johnson & Jackson, 2010; Weber et al., 2013; Vaahtomeri et al., 2017). For visualization, the LEC borders were stained with VE-cadherin antibody.

8.2.2 RAB-GTPase~~s~~ expression in primary human dermal LECs (unpublished)

We then decided to test the functional relevance of the two distinct vesicular pools of chemokine CCL21, in LECs (Fig. 2A in I). To achieve that, I first wanted to characterize the molecular identity of these vesicle types. To that end, I turned to RAB-GTPases, which are well-known markers and master regulators of vesicle types in cells (Fukuda, 2008; Wandinger-Ness & Zerial, 2014; Zhen & Stenmark, 2015). Based on that, using bulk mRNA analysis of cultured primary human dermal LECs, I identified all the expressed RAB-GTPases (Figure 10A, unpublished result).


A
Average mRNA level (normalized)
800
600
400
200
0
RAB1A RAB1B RAB2A RAB2B RAB3A RAB3B RAB3C RAB3D RAB4A RAB4B RAB5A RAB5B RAB5C RAB6A RAB6B RAB6C RAB7A RAB8A RAB8B RAB9A RAB9B RAB10 RAB11A RAB11B RAB12 RAB13 RAB14 RAB15 RAB17 RAB18 RAB20 RAB21 RAB22A RAB23 RAB24 RAB26 RAB27A RAB28 RAB29 RAB30 RAB31 RAB32 RAB33A RAB33B RAB34 RAB35 RAB36 RAB38 RAB39B RAB40AL RAB40B RAB40C RAB42
B
expression
row min
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collLEC
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Rab36
Rab37
Rab38
Rab40b
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C
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0.50
0.75
1.00
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Rab1a
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Rab2a
Rab2b
Rab3a
Rab3b
Rab3c
Rab3d
Rab4a
Rab4b
Rab5a
Rab5b
Rab5c
Rab6a
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Rab32
Rab33b
Rab34
Rab35
Rab36
Rab37
Rab38
Rab39
Rab40b
Rab40c
Rab42
Rab43
Rab44
Cap Prolif
Ptx3 Cap
Cap
Col
Valve

Figure 10: Bulk-mRNA sequencing of cultured LECs and reanalysis of single-cell mRNA sequencing results: **(A)** Bulk-mRNA analysis of RAB-GTPase expression in primary human dermal lymphatic endothelial cells (LECs). The data represents 3 independent experiments with n=8 individual samples. Each data point represents an individual sample. Two out of the three experiments contained untreated LECs, while in the third experiment, the LECs were transduced with mCherry. **(B)** Re-analysis of single-cell mRNA sequencing data of adult mouse mesenteric LECs. In (B), capLEC = capillary LECs, pre.collLEC = pre-collector LECs, collLEC = collector LECs, vLEC = valve LECs, IfnLEC = interferon producing LECs, and prolifLEC = proliferative LECs. The bubble-plot was compiled using online scRNA database interface (Alejandra González-Loyola, 2021), which is related to a study reported by Gonzalez-Loyola et al., 2021 (data ref: NCBI Gene Expression Omnibus: GSE156320). **(C)** Re-analysis of single-cell mRNA sequencing data of mouse dermal LECs *in vivo*. This is the same figure as the published (Fig. EV4B in I). The figure is modified and reused in my thesis under the creative commons CCO 1.0 universal license as per The EMBO Journal policy. In (C), Cap prolif = proliferative capillary LECs, Ptx3 Cap = Ptx3+ capillary LECs, Cap = capillary LECs, Col = collector LECs, and Valve = valve LECs. The bubble-plot was compiled using the database interface published at Mäkinen lab's single cell portal: (Milena Petkova, 2023), which is related to a recent study reported by Petkova et al (data ref: Gene Expression Omnibus, GSE201916) (Petkova et al, 2023). The size of the dots in (B-C) indicates the proportion of LECs expressing the indicated RAB-GTPase and the color indicates the level of expression. Red and cyan arrows in (A-C) indicate the RAB-GTPases used in this thesis for the preparation of mini-library and subsequent colocalization analysis with CCL21ΔC-mCherry and CCL21-mCherry. The RAB-GTPases marked with cyan arrows were also tested via antibody staining of cultured LEC monolayer.

Additionally, we reanalyzed the single cell mRNA sequencing data of *in vivo* adult mouse mesenteric LECs (NCBI Gene Expression Omnibus: GSE156320) (Gonzalez-Loyola et al., 2021) (Figure 10B, unpublished results) and the single cell mRNA data of *in vivo* mouse dermal LECs (data ref: Gene Expression Omnibus, GSE201916) (Petkova et al., 2023) (Figure 10C, which is modified form Fig. EV4 B in I). These databases are available online at (Alejandra González-Loyola, 2021) and (Milena Petkova, 2023), respectively, and, provide an interface for the visualization of single cell mRNA sequencing data of LECs.

Comparison of my bulk mRNA data of cultured primary human dermal LECs (Figure 10A, unpublished data) with the single-cell mRNA sequencing data of *in vivo* mouse mesenteric LECs (Figure 10B, unpublished data) and dermal LECs (Figure 10C, (modified from EV4B in I)), confirmed the expression of RAB-GTPases commonly associated with secretory compartments (Fukuda, 2008; Wandinger-Ness & Zerial, 2014), including RA1A, 2A, 3D, 6A, RAB7A, RAB27A and RAB37 along with many other RABs associated with endosomal vesicles. Based on the expression of many secretion-associated RAB GTPases, it is likely that there might be several secretory routes in LECs. Future experimentation to dissect these routes would be very interesting. Nonetheless, as reflected in my data (Figure 10A-C, this thesis), the

expression level of RAB-GTPases, can vary depending on cell culture or *in vivo* model and also on different types of tissue (dermis vs mesenteries). Based on these lists and the already present knowledge in the literature (Fukuda, 2008; Wandinger-Ness & Zerial, 2014; Zhen & Stenmark, 2015), the RABs which were chosen for testing in LECs using lentiviral overexpression are marked with red and cyan arrows. Red arrows indicate the RABs tested with lentiviral overexpression only, while the RABs marked with cyan arrows were also tested with antibody staining (Figure 10A-C, unpublished data). These RABs are also enlisted here: RAB1A, RAB2A, RAB3D, RAB5C, RAB6A, RAB7A, RAB8A, RAB10, RAB11, RAB13, RAB27, RAB35 and RAB37.

8.2.3 EGFP-RAB GTPase based identity of CCL21 storage vesicles in LECs

To test which of the identified RAB-GTPases colocalized with CCL21 containing vesicles, I sub-cloned a mini-library of fluorophore-tagged RAB-GTPases (EGFP-RABs), which were further transferred into a lentiviral vector, for overexpression in LECs. Using this, I carried out simultaneous transduction of cultured primary human dermal LECs, with lentivirus encoding EGFP-RAB-GTPase and CCL21ΔC-mCherry (Fig. 2D and EV2 A-C in I). Next, I used high-resolution and magnification imaging and identified the co-localization between CCL21ΔC-mCherry and EGFP-RAB-GTPase. The co-localization was quantified via semi-automated analysis, using IMARIS image analysis software. In doing so, I identified the RAB-GTPase identity of the CCL21+ vesicle populations, present in LECs. My results showed that approximately 30% of CCL21ΔC-mCherry+ vesicles, colocalized with EGFP-RAB6A (Fig. 2E in I). Interestingly, these CCL21ΔC-mCherry/EGFP-RAB6A double positive vesicles mostly decorated the multicellular junctions (Fig. 2D and F in I). Previously a study by Martin et al., has reported that RAB6+ vesicles localize to the leading edge of the blood endothelial cells (Martin et al., 2018). My observation showing the peripheral localization of RAB6+ CCL21+ vesicles at the LEC borders, showed that indeed also in LECs, the RAB6+ vesicles constitute the peripheral pool, even in the absence of a leading edge. Other than the RAB6+ pool, 48%-57% of the total CCL21ΔC-mCherry+ vesicles colocalized with EGFP-RAB3D, RAB27A and RAB37 in the perinuclear area (Fig. 2D-F in I). The difference in the RAB identity of the perinuclear and the peripheral pools of CCL21 provided the first proof of their distinct nature.

Moreover, to ascertain the distribution of co-localized CCL21ΔC-mCherry+ vesicles in LECs, I measured the distance of vesicles from the nearest multicellular junction. I found that EGFP-RAB6A/CCL21ΔC-mCherry colocalized vesicles were clearly present much closer to the LEC multicellular junctions (within 0-5 μm), as compared to the EGFP-RAB3D, RAB27A or RAB37 colocalized vesicles (10-55 μm) (Fig. 2F in I). Our previous results showed that most of the DC transmigration, across the lymphatic endothelia, occurs through the LEC multicellular junctions (Fig. 1A-D in I). Moreover, the peripheral pool of LEC chemokine CCL21 was present at the LEC multicellular

junctions (Fig. 2A in I), which has now been identified as the RAB6+ population of CCL21. Due, to the presence of the RAB6+ CCL21+ vesicles at the LEC multicellular junctions, it could be envisioned that these vesicles are exocytosed at these sites, which could act as a guidance cue, to support the transmigration of DCs at the LEC multicellular junctions.

On the other hand, both visual observation and the quantification (Fig. 2F in I), showed that RAB3D, RAB27A and RAB37 in fact represented the same pool of CCL21ΔC-mCherry vesicles, localizing in the perinuclear area. To test that, we generated mCherry-tagged RAB-27A and overexpressed it together with EGFP-RAB3D. Using high resolution spinning disc microscopy, we found almost complete colocalization between both overexpressed RABs. This provided the evidence that indeed RAB3D, RAB27A and RAB37 represent the same population of vesicles (Fig. 3I in I).

Apart from the RAB3D, RAB6A, RAB27A and RAB37, other EGFP-RAB-GTPases, tested for colocalization with CCL21ΔC-mCherry, included RAB1A, RAB2A, RAB5C, RAB7A, RAB8A, RAB10, RAB11, RAB13 and RAB35. These RABs showed comparatively weaker colocalization with CCL21ΔC-mCherry (between 0-11%) (Fig. EV2 A-G in I). As RAB3D, RAB6A, RAB27A and RAB37 represented majority of the peripheral and perinuclear chemokine CCL21 storage vesicles, I primarily focused on these RABs. Nonetheless, intriguingly, I found that EGFP-RAB8A, highlighted high-intensity regions on the plasma membrane, at the LEC multicellular junctions. However, due to the plasma membrane localization, the signal intensity of EGFP-RAB8A was spread all over the cell surface, including high-intensity patches, which made the automatic detection of colocalization with CCL21ΔC-mCherry cumbersome. To overcome this, I manually estimated the colocalization between CCL21ΔC-mCherry vesicles and the EGFP-RAB8A high intensity vesicles (Fig. EV2 H-I in I). I found that, about 7% of the total CCL21ΔC-mCherry vesicles colocalized with EGFP-RAB8A vesicles (Fig. EV2 J in I). More interestingly, localization of these double positive vesicles was similar to the EGFP-RAB6A/CCL21ΔC-mCherry vesicles, i.e. closer to the multicellular junctions, as shown by the quantification of distance from the closest multicellular junction (Fig. EV2 H-K in I). We, therefore, wondered if RAB8A and RAB6 partner together, to maintain the peripheral pool of chemokine CCL21, in LECs. To support this hypothesis, a previous report had indicated, that in HeLa cells, RAB8 and RAB6, together with MICAL3, cooperate to control the docking and fusion of exocytic vesicles (Grigoriev et al., 2011).

Next, I tested if my RAB-GTPase co-localization results also recapitulated with full length CCL21. I confirmed that lentiviral co-overexpression of full length CCL21-mCherry with any of the EGFP-RAB3D, RAB27A, RAB37 or RAB6A, showed similar colocalization percentages and distances from the nearest multicellular junction, as

with CCL21ΔC-mCherry (Fig EV3 A-E in I). At this stage, we were sure that most of the CCL21, is packaged and stored in RAB3D, RAB27A, RAB37 and RAB6A positive vesicles and that the RAB6A population represented the peripheral pool of chemokine CCL21, in LECs. Furthermore, since the RAB6+ pool of CCL21 was present at the LEC multicellular junctions where most of the DC transmigration occurred, I ascertained its identity by antibody staining of endogenous RAB6A, in LECs overexpressing CCL21ΔC-mCherry. I found that like the result showing EGFP-RAB6A/CCL21ΔC-mCherry colocalized vesicles, the antibody stained RAB6 also showed similar peripheral localization of endogenous RAB6 with overexpressed CCL21ΔC-mCherry (Fig. 2H in I). Both EGFP-RAB6A and RAB6 staining also highlighted the Golgi compartment. This is in line with the already present knowledge that RAB6+ vesicles are derived from the Golgi apparatus and facilitate the transport of material between Golgi, endoplasmic reticulum, plasma membrane and endosomes (Jasmin et al., 1992; Martinez et al., 1994; Martinez et al., 1997; Girod et al., 1999; White et al., 1999; Opdam et al., 2000; Young et al., 2005; Del Nery et al., 2006; Utskarpen et al., 2006). Since, siRNA mediated knockdown can be used to hinder the translation of target genes (Dana et al., 2017), transfection with siRAB6 would thereby reduce RAB6 levels in LECs. Using this strategy, I confirmed the specificity of the RAB6 antibody. To that end, I transfected cultured LECs with siRAB6 and observed a complete loss of immunofluorescence signal (Fig. EV5 G in I). Furthermore, to quantify the efficiency of siRAB6 mediated knock-down of RAB6, I performed qPCR and observed loss of mRNA level in LECs transfected with siRAB6 (Fig. EV5 F in I). Therefore, I could trust the antibody staining of RAB6 and the siRNA mediated knock-down approach of RAB6 in cultured primary dermal LECs.

8.2.4 Ultrastructure of CCL21 storage compartments in LECs

To further confirm the identity of the perinuclear and peripheral pools of chemokine CCL21, I used transmission electron tomography and lentiviral overexpression of either CCL21ΔC-mCherry, full length CCL21-mCherry, EGFP-RAB6A or EGFP-RAB27A. Intriguingly, I found that both CCL21ΔC-mCherry and CCL21-mCherry decorated dense-core granules, which were also seen with the marker of perinuclear vesicles EGFP-RAB27A (Fig. 3A-C and S3A in I). Interestingly, RAB27A+ vesicles have previously been associated with a structurally unique, “dense core” characteristic feature (Tsuboi & Fukuda, 2006). My results recapitulated the presence of dense-core RAB27+ vesicles in LECs. Apart from that, CCL21ΔC-mCherry and CCL21-mCherry also localized to “hollow” low-electron density vesicles, in the periphery of the LECs, and especially near the multicellular junctions. This population of vesicles was also seen with EGFP-RAB6A (Immunogold anti-EGFP staining) (Fig. 3A-C and S3A in I), and represented the Golgi-derived direct secretory vesicles (Grigoriev et al., 2007; Miserey-Lenkei et al., 2010; Fourriere et al., 2019). Apart from that, CCL21ΔC-mCherry and CCL21-mCherry were present in the multivesicular bodies (MVB), which

were not highlighted with either EGFP-RAB3D or RAB27A (Fig. 3C in I). MVBs represent the late endosomal compartment which matures into lysosomes and causes degradation of the cargo. However, MVBs can also fuse back to the plasma membrane and release exosomes (Huotari & Helenius, 2011). In short, my results from electron tomography experiment support the presence of CCL21 in distinct pools: the Golgi derived-RAB6+ secretory vesicles and RAB27+ dense core vesicles and the MVBs.

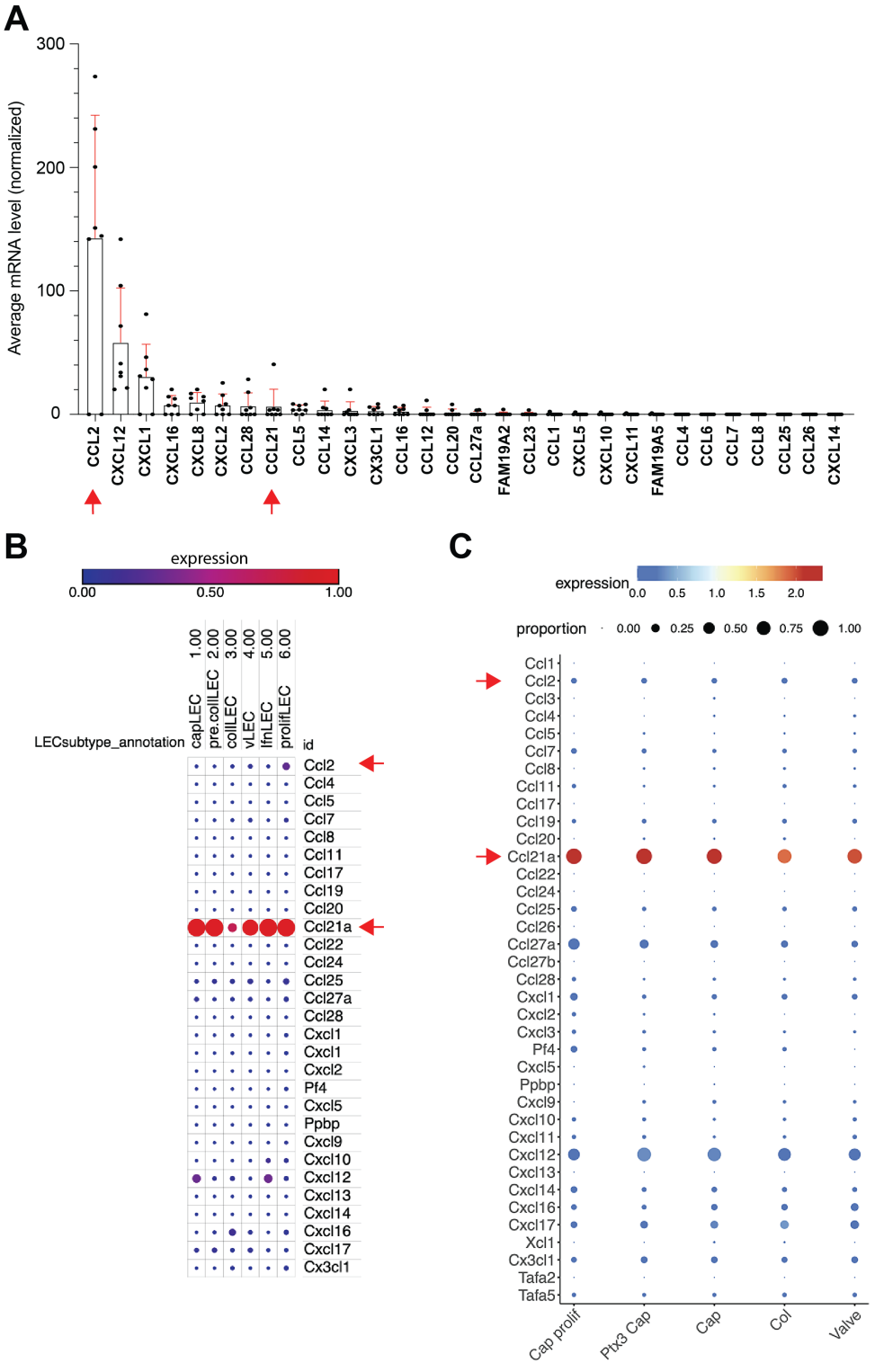
8.2.5 Characterization of late endosomal/lysosomal pool of CCL21ΔC-mCherry

To verify the localization of CCL21 to the late-endosomal/lysosomal compartments in LECs overexpressing CCL21ΔC-mCherry, I co-stained the common markers of late-endosomal vesicles: LAMP3 (Kobayashi, 2000) and RAB7 (Fig. 3D-E and G in I), or for the lysosomal compartments LAMP1 (Cheng et al., 2018) and RAB7 (Fig. 3F and H in I). RAB7 was used in both cases since it is known to be associated with both late endosomes and lysosomes (Bucci et al., 2000). Upon analysis I observed 5.6%-7.5% colocalization between CCL21ΔC-mCherry+ vesicles and the markers of late endosomes and lysosomes. These results confirmed that apart from the peripheral RAB6+ direct secretory pool and the perinuclear dense-core RAB3D/27A+ pool, the chemokine CCL21 is also diverted to the late endosomal/lysosomal compartments. However, this population represented comparatively a minority of total CCL21 present in the LECs. It is possible that the overexpression of CCL21ΔC-mCherry could possibly cause its localization to the late endosomal/lysosomal compartments for degradation.

8.3 RAB6+ and RAB3D/27A+ vesicles represent general secretory route in LECs

8.3.1 Chemokines expressed in LECs

Since endogenous chemokine CCL21 is down-regulated in primary cultured LECs (Wick et al., 2007), my results showing the localization of CCL21ΔC-mCherry or CCL21-mCherry to the EGFP- RAB6A, RAB3D and RAB27 vesicles, were obtained using the overexpression of these proteins in LECs. Now, I wanted to verify if these identified RAB-GTPase compartments indicated a general route for the storage and secretion of other endogenous chemokines. To find out which other chemokines besides CCL21 are expressed in LECs, I carried out bulk-mRNA sequencing of cultured primary human LECs. This is the same dataset as used for the RAB-GTPases in (Figure 10A, unpublished results/this thesis).


A
Average mRNA level (normalized)
300
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CXCL8
CXCL2
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CCL5
CCL14
CXCL3
CX3CL1
CCL16
CCL12
CCL20
CCL27a
FAM19A2
CCL23
CCL1
CXCL5
CXCL10
CXCL11
FAM19A5
CCL4
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Ccl25
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Cxcl5
Ppbp
Cxcl9
Cxcl10
Cxcl12
Cxcl13
Cxcl14
Cxcl16
Cxcl17
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proportion
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Ccl8
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Ccl19
Ccl20
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Cxcl13
Cxcl14
Cxcl16
Cxcl17
Xcl1
Cx3cl1
Tafa2
Tafa5
Cap prolif
Ptx3 Cap
Cap
Col
Valve

Figure 11: Bulk-mRNA of cultured LECs and reanalysis of single-cell mRNA sequencing results: (A) Bulk-mRNA analysis of chemokine expression in primary human dermal lymphatic endothelial cells (LECs). The data represents 3 independent experiments with n=8 individual samples. Each data point represents an individual sample. Two out of the three experiments contained untreated LECs, while the cells in the third experiment were transduced with mCherry. This dataset is from the same experiments as shown in (Figure 10A, this thesis). **(B)** Re-analysis of single-cell mRNA sequencing data of adult mouse mesenteric LECs. In the bubble plot capLEC = capillary LECs, pre.collLEC = pre-collector LECs, collLEC = collector LECs, vLEC = valve LECs, IfnLEC = interferon producing LECs, and prolifLEC = proliferative LECs. The bubble-plot was compiled using the database interface published at (Alejandra González-Loyola, 2021), which is related to a study reported by Gonzalez-Loyola et al., 2021 (data ref: NCBI Gene Expression Omnibus: GSE156320). **(C)** Re-analysis of single-cell mRNA sequencing data of mouse dermal LECs *in vivo*. In (C), Cap prolif = proliferative capillary LECs, Ptx3 Cap = Ptx3+ capillary LECs, Cap = capillary LECs, Col = collector LECs, and Valve = valve LECs. The bubble-plot was compiled using the database interface published at (Milena Petkova, 2023), which is related to a recent study reported by Petkova et al (data ref: Gene Expression Omnibus, GSE201916) (Petkova et al, 2023).
The size of the dots in (B-C) indicates the proportion of LECs expressing the indicated chemokine and the color indicates the level of expression. The red arrows indicate the chemokines CCL21 and CCL2, which are explored in this thesis.

My results showed that the mRNA level of chemokines CCL2 and CXCL12 were amongst the most abundant in cultured cells (Figure 11A, unpublished result, this thesis). However, antibody staining of endogenously expressed CCL2, in fixed LECs, did not show immunofluorescence signal (data not shown). This meant that, probably because of post transcriptional regulation, the chemokine CCL2 protein is not expressed in substantial amounts, in uninflamed LECs. A previous study showed that CCL2 expression can be induced in LECs upon inflammation (Ohta et al., 2000). In agreement to that, treatment of LEC with TNF-α, 24 h prior to fixation, resulted in a strong immunofluorescence signal of endogenous CCL2, present in vesicles (data not shown). To ascertain the localization of the second most abundant chemokine (CXCL12), in my bulk mRNA analysis (Figure 11A, unpublished result, this thesis), I stained fixed LEC monolayer for CXCL12 (unpublished results, Vaahtomeri lab). My preliminary staining results indicated that CXCL12 mostly highlighted cellular structures which resembled cell cytoskeleton and microtubules. However, some of the CXCL12 also localized to vesicles, which were dispersed in the cell cytoplasm. Since, the specificity of the CXCL12 antibody staining was questionable, it was not used for further experimentation. However, it is known that LECs secrete CXCL12, which can be immobilized on the LEC surface by binding to heparan sulfate (Pablos et al., 2003). Previous studies have also shown that cutaneous DC can sense CXCL12 via their CXCR4 receptor, which can facilitate their migration (Kabashima et al., 2007). Based on these important functions, it would be interesting to further study the secretory route of CXCL12 in LECs.

Apart from the bulk-mRNA data, to further confirm which chemokines exist *in vivo* in LECs, I reanalyzed the single cell mRNA sequencing data of *in vivo* adult mouse mesenteric LECs (NCBI Gene Expression Omnibus: GSE156320) (Gonzalez-Loyola et al., 2021) (Figure 11B, unpublished results, this thesis) and the single cell mRNA data of *in vivo* mouse dermal LECs (data ref: Gene Expression Omnibus, GSE201916) (Petkova et al., 2023) (Figure 11C, unpublished result, this thesis). These databases are the same as the ones referred for RAB-GTPases in section 8.2.2 above. Re-analysis done via these databases showed that in *in vivo* mouse mesenteric and dermal LECs, CCL21 is the most highly expressed chemokine in homeostasis. Chemokines CCL2 and CXCL12 were also detected in these plots (Figure 11B-C, unpublished results, this thesis), but were less expressed as compared to CCL21 under homeostatic conditions in *in vivo* dermal and mesenteric LECs. As reported in a previous study, the expression of many proteins including inflammation associated chemokines, can be induced upon inflammation, which can facilitate the migration of DCs (Vigil, 2011). It is possible that CCL2 and CXCL12, amongst other chemokines, showed comparatively higher expression in my bulk-mRNA results (Figure 11A, unpublished results, this thesis), as cell culture conditions, to some extent, represent the inflammatory state. This is also reflected in the cell junctions of cultured LECs, which display "zipper-like" LEC junctions, reported for the inflamed lymphatic capillaries (Yao et al., 2012).

On the contrary, although CCL2 was shown to be highly expressed in my bulk-mRNA result (Figure 11A, unpublished result, this data), I did not see it with antibody staining in un-inflamed LECs (explained above), but CCL2 was enriched only after treatment of cultured LECs with TNF-α. It is possible that despite the increase in mRNA level of CCL2 in cultured LECs, the effect was not translated into the expression of protein, in the absence of an inflammatory stimulus. However, based on a previous study (Vigil et al., 2011) and my result (Figure 11, unpublished results, this thesis), besides chemokine CCL21 and CCL2, many other chemokines can also be secreted by LECs, which might be involved in the guidance of DCs. The figure below (Figure 12, unpublished data, this thesis), summarizes the known chemokines and their receptors expressed in LECs and DCs in steady state and upon inflammation, i.e. the possible ligand-receptor pairs, involved in the DC guidance.

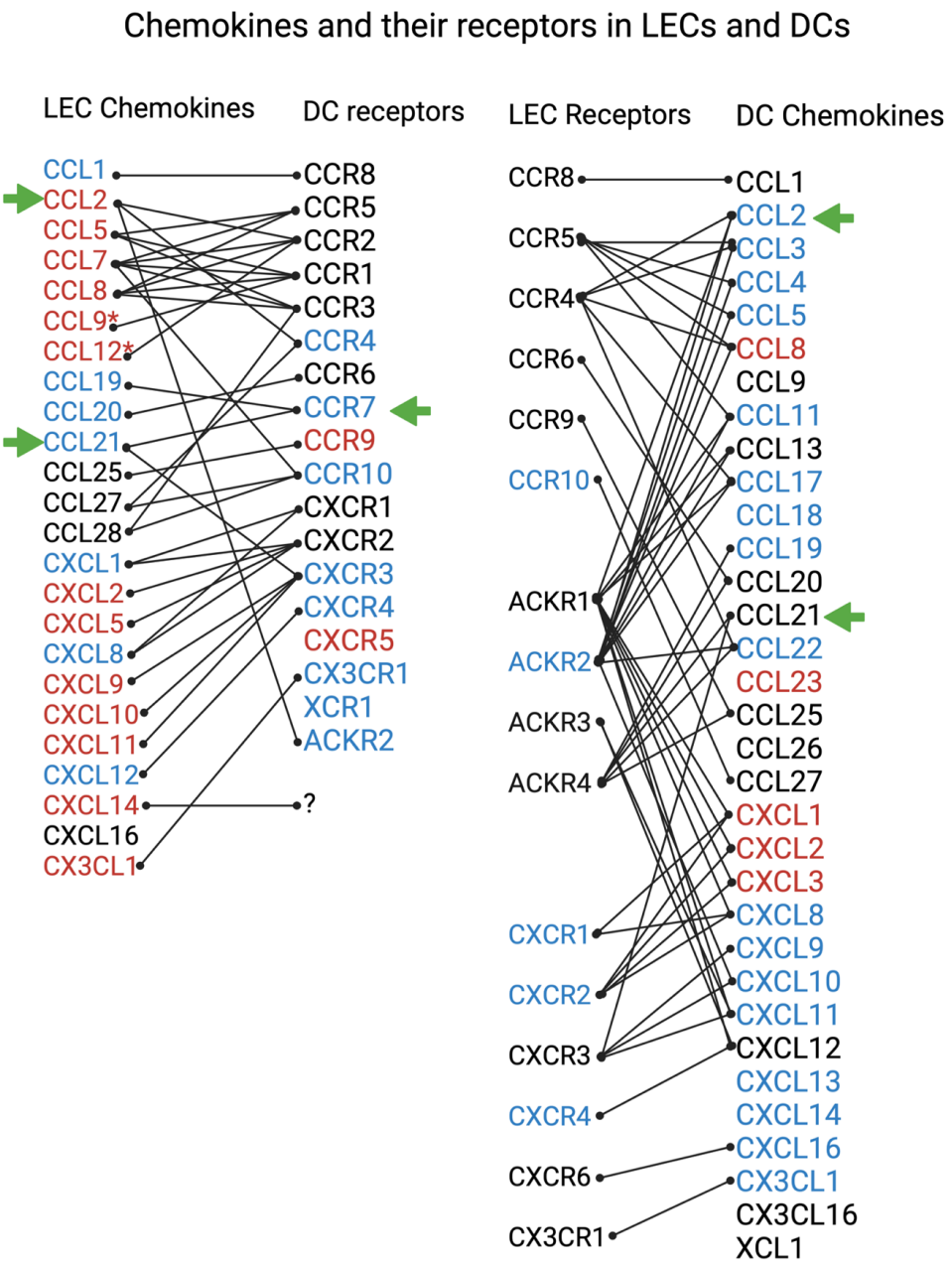

Figure 12: Chemokines and their receptors expressed in lymphatic endothelial cells and dendritic cells: The figure shows the chemokines/receptors expressed in LECs and DCs in steady state (black text), upon inflammation (red text), and the ones expressed in both inflammation and steady state (cyan text). The green arrows in the figure show the chemokines/receptors explored in this thesis. Apart from the known CCL21/CCR7 axis (Forster et al., 1999; Ohl et al., 2004; Vaahtomeri et al., 2017), other types of chemokine signaling pathways might provide guidance to the migrating DCs. The black connecting

lines in the figure show the possible chemokine/ligand pairs. This figure was created from the information available in the following studies/reviews (Mohamadzadeh et al., 1996; Vicari et al., 1997; Rubbert et al., 1998; Cella et al., 1999; Nardelli et al., 1999; Garcia-Lopez et al., 2001; Nibbs et al., 2001; Oppenheim et al., 2002; Rappert et al., 2002; Katou et al., 2003; Zhao et al., 2003; Mantovani et al., 2004; Real et al., 2004; Rot & von Andrian, 2004; Schutyser et al., 2005; Tabata et al., 2005; Piqueras et al., 2006; Zhang et al., 2006; Heinzel et al., 2007; Kabashima et al., 2007; Pegu et al., 2007; McKimmie et al., 2008; Pallandre et al., 2008; Ricciardi et al., 2008; Wick et al., 2008; Zimmerman et al., 2008; Dorner et al., 2009; Salogni et al., 2009; Jin et al., 2010; Neusser et al., 2010; Seth et al., 2011; Sisirak et al., 2011; Vigil, 2011; Cha et al., 2012; Malhotra et al., 2012; Teijeira et al., 2012; Zhuo et al., 2012; Choi et al., 2013; Das et al., 2013; Haemmerle et al., 2013; Johnson & Jackson, 2013; McKimmie et al., 2013; Ajram et al., 2014; Ford et al., 2014; Lee et al., 2014; Ulvmar et al., 2014; Blatt et al., 2016; Chang & Chen, 2016; Sun et al., 2016; Mohan et al., 2017; Rump et al., 2017; Ford et al., 2018; Hornick et al., 2018; Kroczek et al., 2018; Soler-Cardona et al., 2018; Farnsworth et al., 2019; Karnezis et al., 2019; Rapp et al., 2019; Saxena et al., 2019; Kumaravel et al., 2020; Murcia et al., 2020; Shi et al., 2020; Xiang, 2020; Di Sapia et al., 2021; Gonzalez-Loyola et al., 2021; Liu et al., 2021; Ozga et al., 2021; Roy et al., 2021; Vaahtomeri et al., 2021; Chang et al., 2022; Gowhari Shabgah et al., 2022; Si et al., 2022; Arroz-Madeira et al., 2023; Crawford & Volkman, 2023; Korbecki et al., 2023; Milena Petkova, 2023). Expression and receptor ligand pairing has been done if at least one study reported it. The figure was created using BioRender.com.

8.3.2 general secretory route in LECs

Next, I verified if the identified RAB6A, RAB3D and RAB27 vesicles represented a more general secretory route in LECs. I confirmed that with 1) staining of endogenous CCL2, (2) over expression of CCL21 and, (3) overexpression of CCL21 signal peptide fused to mCherry (sp-mCherry), thus representing a general secretory marker. To produce sp-mCherry, I deleted the DNA sequence of CCL21 using site-directed mutagenesis, leaving behind only the N-terminus of CCL21. For the expression of sp-mCherry in cultured LECs, I transferred it into a lentiviral vector and produced the lentivirus.

Chemokine CCL2 is also known as monocyte attractant protein 1 (MCP-1), which provides chemotactic cues for the migration of monocytes (Matsushima et al., 1989) and other immune cells (Carr et al., 1994; Frade et al., 1997; Gendelman et al., 2009). To verify the colocalization of CCL2 with RAB-GTPases, I used lentiviral overexpression of EGFP-RAB3D, EGFP-RAB27A or EGFP-RAB6A, in cultured dermal LECs, co-transduced with sp-mCherry. To induce the expression of CCL2 in LECs, I treated the monolayer with TNF-α for 24 h, prior to the fixation. I found that similar to CCL21ΔC-mCherry, the sp-mCherry and CCL2 both colocalized with EGFP-RAB6A, very close to the multicellular junctions (Fig. EV4A in I). Moreover, the sp-mCherry colocalized with EGFP-RAB3D and EGFP-RAB27A in the perinuclear area, similar to CCL21ΔC-mCherry. However, the perinuclear CCL2+ vesicles, showed lesser

colocalization with EGFP-RAB3D and EGFP-RAB27A, as compared to CCL21. Some of the CCL2+ vesicles did not colocalize with any of the overexpressed RAB-GTPases and therefore remained unidentified (Fig. EV4A in I). Nonetheless, I also, saw triple positive vesicles containing EGFP-RABs, sp-mCherry and CCL2. Moreover, I also confirmed the presence of the peripheral RAB6 positive pool of CCL2. For that, I stained both endogenous CCL2 and RAB6, in LECs treated with TNF-α. I observed the presence of RAB6/CCL2 double positive vesicles near the LEC junctions (Fig. 2I in I). This indicated that that RAB3D/RAB27A and RAB6 represent general compartments, where secretory cargo is stored, in LECs.

To confirm if both chemokines CCL2 and CCL21ΔC-mCherry colocalized together in the same compartments, I overexpressed CCL21ΔC-mCherry in cultured LECs, treated with TNF-α, and stained endogenous CCL2. As expected, the overexpressed CCL21ΔC-mCherry colocalized robustly with endogenous CCL2, both in perinuclear and peripheral areas, indicating that both chemokines are probably secreted through the same secretory route (Fig. 2G in I). Conclusively, my results showed that in LECs, both chemokines CCL21 and CCL2 containing vesicles, are trafficked to the multicellular junctions. Due to this it can be envisioned that the RAB6+ route represents a general secretory pathway.

8.4 CCL21 containing RAB6+ vesicles localize to the LEC multicellular junctions in mouse ear pinna dermis *in vivo*

Next, I confirmed my finding about the multicellular junction localization of RAB6A+ CCL21 containing vesicles in mouse ear pinna dermis lymphatic vessels. To this end, I co-stained mouse ear dermis with antibodies against mouse-specific CCL21, RAB6 and LYVE1 (lymphatic capillary marker) or VE-cadherin (cell junction marker). My staining showed, the lymphatic collectors displaying continuous VE-cadherin, while the lymphatic capillaries exhibited discontinuous VE-cadherin signal (Baluk et al., 2007). This continuous VE-cadherin in the lymphatic collectors could be thought of as the “zipper-like” junctions, while the discontinuous VE-cadherin could represent the “button-like” junctions, in homeostasis, as reported in previous studies (Baluk et al., 2007; Arasa et al., 2021). I found that, in mouse ear pinna dermal lymphatics, 39% of CCL21+ vesicles were also positive for RAB6 in the pre-collectors (Fig. 2M in I) and 44% in the capillary LECs (Fig. 2N in I). This was similar to the co-localization percentage observed in the cultured primary human dermal LECs, overexpressing EGFP-RAB6A and CCL21ΔC-mCherry (Fig. 2D-E in I). It is known that the LEC button-like junctions could transition into zipper-like confirmation, upon inflammation (Yao et al., 2012). This is also seen upon culturing primary human dermal LEC, which exhibit continuous VE-cadherin staining. Therefore, my cell-culture results more closely represent the inflamed lymphatic capillaries *in vivo*.

Furthermore, to show that the RAB6+ CCL21+ vesicles existed near the LEC junctions *in vivo*, as in my cell-culture findings (Fig. 2F in I), I compared the distribution of the colocalized versus the non-colocalized CCL21+ vesicles, with respect to their distance from the nearest cell junction. My analysis showed that here once again, the RAB6+ CCL21+ vesicles are enriched within 5 μm of the multicellular junction, in case of pre-collector (Fig. 2K and N in I). However, in case of discontinuous VE-cadherin staining of the blind-ended lymphatic capillary LECs, it was not possible to correctly ascertain the exact position of the multicellular junctions. Therefore, in this case I measured the distance from the nearest VE-cadherin signal, i.e. the cell border. I found that here too, the majority of RAB6+ CCL21+ vesicles were closest to the nearest cell border (within 2 μm) (Fig. 2L and P in I). Furthermore, the non-colocalized CCL21+ vesicles were much sparse near the cell junctions, in both the pre-collectors and capillaries (Fig. 2N-P in I). This is in-line with my cell culture findings, where RAB6+ pool of CCL21 is closest to the cell junctions (peripheral pool), while the RAB3/27+ CCL21+ vesicles are present in the cell body (peri-nuclear pool) (Fig. 2F in I).

Existing evidence has shown that the CCR7/CCL21 signaling is required to drive the transmigration of DCs, into the lymphatic vessel lumen (Vaahtomeri et al., 2017). In conjunction to that, we observed that DCs preferentially transmigrated through the LEC multicellular junctions (Fig. 1A-B in I). Together with these, my result, showing the enrichment of the RAB6+ pool of CCL21 at the discontinuously stained VE-cadherin+ LEC junctions indicates that the RAB6+ population of CCL21 is also relevant under homeostatic conditions (button junctions), to drive DC transmigration.

8.5 CCL21 containing RAB6+ vesicles are targeted and preferentially exocytosed at the LEC multicellular junctions

8.5.1 RAB6+ vesicles are delivered to the LEC multicellular junctions

To study how the RAB6+ CCL21 containing vesicles arrive at the multicellular junctions, I carried out lentiviral overexpression of EGFP-RAB6A in cultured primary human dermal LECs. I then followed the trafficking of RAB6A+ vesicles with the help of fluorescent live imaging. I observed that RAB6A+ vesicle trajectories initiated from the perinuclear area and targeted the multicellular junctions (Fig. 4A and Movie EV9 in I). Additionally, I noticed that most of the RAB6+ vesicles, after arriving at the multicellular junctions, did not leave these sites, while some vesicles disappeared (exocytosed) (Movie EV9 in I). However, a small minority of RAB6+ vesicles travelled back towards the perinuclear area (Movie EV9 in I). Nonetheless, the migratory routes of RAB6+ vesicles intriguingly looked quite similar to microtubules (Fig. 4A in I). To confirm that, I stained the microtubules, in LECs. Indeed, my results showed that most of the microtubules connected the multicellular junctions with the perinuclear area

(Fig. 4C in I). Moreover, to confirm if RAB6+ vesicles are associated with the microtubules in LECs, I co-stained RAB6 and microtubules in cultured LECs overexpressing CCL21ΔC-mCherry. I found that indeed the RAB6+ CCL21ΔC-mCherry+ vesicles nicely decorated the microtubules and most of these colocalized vesicles existed near the LEC multicellular junctions (Fig. 4B in I). This finding supported the fact that the RAB6+ vesicles use microtubules to reach the multicellular junctions. This is in compliance with several studies which have already shown the involvement of microtubules in the trafficking of the RAB6+ vesicles, in other cell types and, also the CCL21+ vesicles, in LECs (Grigoriev et al., 2007; Vaahtomeri et al., 2017; Fourriere et al., 2019).

To further understand if the newly formed microtubules are targeted to the LEC multicellular junctions, we carried out lentiviral transduction of cultured LECs, with EB3-mCherry, which marked the growing end (+end) of the microtubule (Komarova et al., 2009). With the help of fluorescent live microscopy we followed the growth of newly forming microtubules. We found that most of the microtubules in fact started growing from the perinuclear area, radially towards the LEC bicellular and multicellular junctions (Movie 10 in I). The EB3-mCherry+ comets upon reaching the bicellular junction continued to travel, on the cell borders, towards the multicellular junction (Fig. 4D and Movie 10 in I). Upon reaching the multicellular junctions, the EB3-mCherry+ comets, disappeared, indicating the stabilization of the microtubules (Fig 4D-E and Movie 10 in I). Altogether these results showed that, indeed, microtubules are preferentially targeted towards the LEC multicellular junctions, and that they transport the RAB6+ CCL21 containing vesicles to these sites. Based on these results, it is possible that the RAB6 vesicles arrive to the multicellular junctions, simply by delivery to the end of the microtubules.

8.5.2 RAB6+ pool of CCL21 is preferentially exocytosed at the LEC multicellular junctions

To check if the RAB6+ CCL21 containing vesicles are secreted at the LEC multicellular junctions. I did lentiviral overexpression of CCL21ΔC-mCherry in cultured LECs. The thin nature of the LECs allowed the use of live epifluorescence microscopy, to visualize the disappearance of CCL21ΔC-mCherry vesicles (i.e. the exocytosis events). The LEC cell junctions were stained with non-blocking fluorophore-tagged anti VE-cadherin antibody prior to imaging. My previous results (discussed above) had confirmed that the RAB6+ pool of CCL21ΔC-mCherry existed closest to the cell junctions. Therefore, I quantified the disappearance of vesicles near the VE-cadherin-stained cell borders (i.e. 7 μm from the junctions, inside the cell body) (Fig. 2B in I). I observed that, indeed most of LEC chemokine CCL21ΔC-mCherry vesicle disappearance events (exocytosis) occurred at the multicellular junctions (i.e. within 0-5 μm) (Fig. 2B-C in I). The number of observed CCL21ΔC-mCherry exocytosis events reduced considerably while

moving, along the bi-cellular junction, further away from the multicellular junctions (i.e. 10-50 μm) (Fig. 2C in I). This showed that LEC multicellular junctions are not only the sites where RAB6+ CCL21 containing vesicles station but are also the places where the chemokine CCL21 is preferentially exocytosed. Previous studies have shown that, upon inflammation, the venular BECs, present BEC-expressed chemokine CXCL1 at the cell surface to promote neutrophil migration and, also, present neutrophil-expressed CXCL2 at BEC junction, via binding to the ACKR1 receptor to promote transmigration (Girbl et al., 2018). These, altogether, allowed the attraction of the neutrophils to the BEC junctions (Girbl et al., 2018). Apart from that, another study showed that actin-associated intra-BEC chemokine CCL2 containing vesicles were locally present at the sites where T-cells interact with BECs (Shulman et al., 2011). My results (this thesis) showed the first proof of the exocytosis of chemokine at the site of DC transmigration. It can be envisioned that the approaching DCs sense the targeted release of CCL21 at the LEC multicellular junctions, are preferentially guided to these sites, as compared to the rest of the LEC surface.

A previous report showed that DC-LEC contact can induce Ca^{+2} signaling in LECs, which causes the on-demand/conditional exocytosis of some of the CCL21ΔC-mCherry+ vesicles (Vaahtomeri et al., 2017). To study if DC-LEC contact can trigger the exocytosis RAB6+CCL21+ peripheral pool of CCL21, we loaded LPS activated DCs, on top of LEC monolayer overexpressing CCL21ΔC-mCherry. With live microscopy, we observed no change in the exocytosis of peripheral CCL21 (up to 7 μm from the cell junction inside the cell body – see figure 2B in I), despite the DC-LEC contact (Movie EV8 in I). Additionally, I also treated the LEC monolayer overexpressing CCL21ΔC-mCherry, with BAPTA-AM, which is a known calcium ion chelator (Vaahtomeri et al., 2017). This treatment did not also affect the exocytosis of CCL21ΔC-mCherry, at the LEC borders (7 μm from the cell junction) (Fig. EV1P in I). These results showed that the RAB6+ peripheral pool of CCL21 is not under the control of Ca^{+2} signaling but rather it is constitutively exocytosed. The targeted exocytosis of the RAB6+ pool of chemokine CCL21, at the LEC multicellular junctions, could therefore result in the high concentration of chemokine CCL21 at these sites. This might allow the guidance of DCs to the LEC multicellular junctions and their eventual transmigration.

On the other hand, the previously reported, conditionally secreted pool of chemokine CCL21 (Vaahtomeri et al., 2017), may therefore represent the RAB3D/RAB27A+ vesicles, present in the cell body. Previous studies have shown that RAB27+ dense core secretory granules, described in other cell types, are usually calcium sensitive (Vitale et al., 2005; Lopes da Silva & Cutler, 2016; McCormack et al., 2017; Nass et al., 2021). In the BECs, a specialized secretory granule population called the Weibel-Palade bodies (WPBs), had been reported (Weibel & Palade, 1964). These granules store adhesion receptors von-Willebrand factor (VWF) and P-selectin, amongst other cargo, whose exocytosis can be evoked in an on-demand Ca^{2+}-dependent manner (Wagner et

al., 1982; Bonfanti et al., 1989; Lowenstein et al., 2005; Criado Santos et al., 2019). The RAB-GTPase identity of WPBs includes RAB27A, RAB3B and RAB3D (Zografou et al., 2012; Kat et al., 2021). This suggests similarity between the WPBs and the RAB3/27A+ CCL21+ pool of CCL21 in LECs.

Taken together, my results and the previously published reports indicate that, the exocytosis of the distinct pools of chemokine CCL21: the RAB6+ pool, the RAB3/27+ pool is regulated via different control mechanisms. Conclusively, our results here provide the first characterization of the RAB6+ constitutively secreted pool of chemokine CCL21ΔC-mCherry, which could be essential in marking the preferential site of transmigration (this thesis). Whereas the calcium sensitive RAB3/RAB27+ conditional pool might be needed to retard the motion of the arriving DCs and could cause their arrest at the LEC surface (Vaahtomeri et al., 2017) . It is possible that the exocytosis of CCL21 from the distinct pools of CCL21 help in the maintenance of the chemokine gradient in the surrounding tissues (Weber et al., 2013), and provide additional control over the transmigration of DCs and determination of the preferential transmigration site.

Moreover, it is expected that the identified chemokine CCL21+ vesicles, most likely contain other guidance cues besides CCL21, which facilitate the transmigration of DCs. These cues could consist of for example, CX3CL1, which was reported to facilitate DC trafficking in inflamed lymphatics (Johnson & Jackson, 2013). My co-localization results (Fig. 2I and EV4A in I), show that chemokine CCL2 is also transported in RAB6+ and RAB3/27+ vesicles, and could possibly be secreted via the same secretory route. Apart from this, LECs also express many other chemokines (Figure 12, unpublished data, this thesis). It is possible that some of those chemokines are also secreted together with CCL21 and fine-tune the guidance of DCs into the lymphatics. Future, studies aimed at characterizing the content of RAB6+ and RAB3/27+ vesicle pools of chemokine CCL21, would provide further details into how LECs regulate the transmigration of DCs.

Conclusively, based on my results, I propose a model where chemical and biomechanical mechanisms act synergistically to orchestrate the transmigration of DCs. Previous studies have reported that in homeostasis, the DC-LEC interactions occur via the hyaluronan and LYVE-1 (Johnson et al., 2017; Stanly et al., 2020). Whereas in inflammation, the LECs and DCs upregulate the expression of integrins and their ligands on their surfaces, which allow increased interactions mediated via the LFA/ICAM and VLA/VCAM contacts between the DC and LEC (Johnson et al., 2006; Johnson & Jackson, 2010; Teijeira et al., 2013; Arasa et al., 2021). These increased interactions between the LEC and DCs could be necessary for the arrest of DCs at the site of transmigration. However, future experimentation is needed to study whether LECs upregulate integrins or their ligands locally at the multicellular

junctions, i.e. the site of DC transmigration. Furthermore, it is known that in the blood endothelia, the arrest of leukocyte on the blood vessel wall can induce the influx of Ca^{+2} ions into the BECs (Huang et al., 1993). In BECs, the increase in the Ca^{+2} level, can lead to the activation of the myosin light chain kinase and RhoA, which via the downstream activation of ROCK can cause the modelling of the F-actin, induce contractility and, thus, provide the biomechanical forces required for the opening of the LEC junctions (Carbajal et al., 2000; Muller, 2003) (see section 4.5). Existing evidence has shown that DC-LEC contact can induce calcium signaling in LECs (Vaahtomeri et al., 2017). It is possible that the changes in LEC calcium levels could induce contractility in LECs and allow the opening of the LEC junctions during the DC transmigration event. However, future experimentation is needed to study what mediates the opening of the LEC junctions and the ways by which it can be controlled. This knowledge would provide additional tools to control the entry of DCs into the lymphatics and, thus the activation of T-lymphocytes in the lymph nodes.

8.6 RAB6 is required for the exocytosis of CCL21ΔC-mCherry at the DC transmigration site (LEC multicellular junctions)

Our results showed that CCL21ΔC-mCherry, is transported to the LEC borders in RAB6+ vesicles (Fig. 4B in I). To confirm if RAB6 is required for the exocytosis of CCL21ΔC-mCherry+ vesicles in LECs, I carried out siRNA mediated silencing of RAB6, in LEC monolayer, overexpressing CCL21ΔC-mCherry. Inhibiting RAB6 could possibly result in blocking the exocytosis of the peripheral pool of CCL21ΔC-mCherry. To ascertain the efficiency of siRNA mediated knock-down, I used qPCR and immunofluorescence staining of RAB6 protein in siCTRL and siRAB6 treated LECs (Fig. EV5F-G in I). My results indicated that upon siRAB6, there was a total ablation of RAB6 immunofluorescence signal in cultured LECs (Fig. EV5G in I). My quantitative PCR results also showed substantial drop in mRNA level, upon siRAB6 transfection of cultured LECs (Fig. EV5F in I). Next, with the help of fluorescent live imaging, we showed that siRAB6 treatment ablated the exocytosis of chemokine CCL21ΔC-mCherry at the LEC junctions (Fig. 6A-B in I). This simultaneous transduction/transfection of primary cultured LECs was a new protocol in the lab which I rigorously optimized prior to experimentation. Moreover, siRAB6 also induced a build-up of CCL21ΔC-mCherry+ vesicles specifically at the LEC multicellular junctions and to some extent also in the rest of LEC body (Fig. 6C-F in I). This build-up of CCL21ΔC-mCherry+ vesicles upon siRAB6 treatment could be explained by their failure to be exocytosed. This is possible because RAB6 interacts with other docking factors (this thesis and Fourriere et al., 2019; Grigoriev et al., 2007; Grigoriev et al., 2011; Lansbergen et al., 2006), which allows the exocytosis of the cargo (CCL21). Ablation of RAB6 impaired this process and lead to the accumulation of vesicles at the multicellular junctions i.e. at the normal site of exocytosis.

To test if the ablating the secretion of chemokine CCL21ΔC-mCherry via siRAB6 hindered the transmigration of DCs, we loaded LPS activated DCs on top of LEC monolayer, overexpressing either CCL21ΔC-mCherry or CCL21-mCherry and treated with siRAB6. Using fluorescent live microscopy, we observed a 29.9% drop in the extent of DC transmigration across the LEC monolayer expressing CCL21ΔC-mCherry (Fig. 6K and Movie EV11 in I). However, no difference was observed in the DC transmigration across LECs transduced with CCL21-mCherry. This could be due to the accumulation of full length CCL21-mCherry on the cell culture dish surface, which does not occur *in vivo*, as reported by (Vaahtomeri et al., 2017). Based on this, our model using CCL21ΔC-mCherry, instead of full length CCL21-mCherry, more closely represents the *in vivo* situation. Conclusively, our results show that RAB6 is essential for the exocytosis of CCL21 cargo at the LEC multicellular junctions, because chemokine CCL21 is brought to the LEC multicellular junctions in RAB6+ vesicles (Fig. 2D-E in I). Inhibition of RAB6 could affect the RAB6+ peripheral pool of chemokine CCL21 at the LEC multicellular junctions and therefore hinder the transmigration of DCs at these sites.

8.7 RAB6-interacting protein ELKS is present at the LEC junctions

Our findings so far confirmed that the LEC multicellular junctions are special, since they form the preferential sites of DC transmigration and that occurs because of the secretion of RAB6+ pool of chemokine CCL21, preferentially at these sites. Based on this we questioned what enables the secretion of RAB6+ CCL21 containing vesicles at the LEC multicellular junctions. Previous studies have indicated that the RAB6-interacting proteins, ELKS and RAB8A are required for the docking and fusion of RAB6+ vesicles (Lansbergen et al., 2006; Grigoriev et al., 2007; Grigoriev et al., 2011; Fourriere et al., 2019). Also, a study done in neurons has reported that ELKS captures RAB6+ vesicles and immobilizes them, right before they are secreted (Nyitrai et al., 2020). Based on this, we decided to test if the reason for the preferential targeting of RAB6+ vesicles to LEC multicellular junctions was due to the presence of ELKS at these sites. To this end, we carried out staining of endogenous ELKS, in cultured primary human dermal LECs. We found that ELKS was located at the LEC borders, with highest expression at the multicellular junctions. The ELKS fluorescence signal decayed along the bi-cellular junctions and plateaued at around 10 μm, away from the nearest multicellular junction (Fig. 5A-B in I).

The higher expression of ELKS at the LEC multicellular junctions meant that it could potentially mediate the docking of RAB6+ CCL21 containing vesicles at these sites. Alternatively, it could be envisioned that like reported for neurons (Nyitrai et al., 2020), the ELKS mediated immobilization of RAB6+ vesicles could provide a way for the creation of a chemokine depot, specifically at the LEC multicellular junctions.

Previous studies had reported DC-LEC contact can trigger and on-demand release of chemokine CCL21 in LEC (Vaahtomeri et al., 2017). However, our results (explained in section 8.5.2), had shown that that DC-LEC contact does not affect the exocytosis of the RAB6+ peripheral pool of CCL21 (Movie EV8 in I). Moreover, this pool of CCL21 does not respond to the treatment with calcium chelator BAPTA-AM Fig. EV1P in I). In the light of these results, it is pertinent that the RAB6+ CCL21 containing vesicles constitute a novel pool of chemokine CCL21, which is preferentially localized to the LEC multicellular junctions (Fig. 2D-E and J-P in I), due to the presence of RAB6-interacting protein ELKS at these sites.

8.8 Preferential exocytosis of chemokine CCL21 at LEC multicellular junctions is determined by ELKS

Previous studies had indicated that LL5-β recruits ELKS to the plasma membrane (Lansbergen et al., 2006). This occurs via the binding of LL5-β to phosphatidylinositol (3,4,5)- trisphosphate (PIP3), via the Plekstrin Homology (PH)-domain, located in the C-terminus of LL5-β (Paranavitane et al., 2003; Lansbergen et al., 2006). The plasma membrane bound phosphatidylinositol (3,4,5)-bisphosphate (PIP2) is phosphorylated via phosphoinositide 3-kinase (PI3K), to generate PIP3 (Cantley, 2002). Since the LL5-β is dependednt on PI3K (Paranavitane et al., 2003; Lansbergen et al., 2006), we tested if blocking PI3K activity would affect the localization of ELKS. To do that, we treated LECs with PI-103, which is a known inhibitor of PI3K activity (Bain, 2007). After fixation we carried out immunofluorescence staining of ELKS. We observed that upon inhibition, the fluorescence signal of ELKS was reduced everywhere on LEC borders, including the multicellular junctions. Moreover, the effect was more pronounced with higher concentration of the inhibitor (Fig. EV5A-C in I). This result confirmed the dependence of ELKS localization to the LEC borders, on PI3K activity.

Furthermore, to check if ELKS was essential for the exocytosis of CCL21 from the RAB6+ vesicles, I carried out siRNA mediated knock-down of ELKS in cultured primary LEC monolayer. I confirmed the efficiency of siRNA mediated knock-down of ELKS both with immunofluorescence staining of ELKS protein in siControl and siELKS treated LECs and with qPCR (Fig. EV5D-F in I). Upon siELKS treatment, I witnessed a complete loss of ELKS immunofluorescence signal with ELKS antibody. Additionally, I also observed significant loss of ELKS mRNA level with siELKS pool and also with individual siELKS oligos (Fig. EV5 E-F in I). Next, using, fluorescent live imaging, I found that siELKS ablated the exocytosis of CCL21ΔC-mCherry at the LEC multicellular junctions. Accordingly, siELKS treatment also resulted in a build-up of CCL21ΔC-mCherry+ vesicles at the multicellular junctions (Fig. 5E-H in I).

Based on the accumulation of CCL21ΔC-mCherry+ at the LEC multicellular junction, we wanted to confirm if it is the RAB6+ pool of CCL21ΔC-mCherry+ vesicles which accumulated upon siELKS. To test that, I carried out staining of endogenous RAB6 in siELKS treated cultured LEC monolayer, overexpressing chemokine CCL21ΔC-mCherry. I then imaged the samples with high magnification spinning disc microscope. Indeed, the accumulated CCL21ΔC-mCherry+ vesicles, near the LEC multicellular junctions (withing 5 μm), were also positive for RAB6 (Fig. 5I-J in I). However, these CCL21ΔC-mCherry/RAB6 double positive vesicles were not present in the rest of the cell body (Fig. 5J in I). The absence of ELKS at the LEC multicellular junctions impaired the exocytosis of the RAB6+ pool of chemokine CCL21 and caused the accumulation of these vesicles. Based on this we ascertained the relevance of ELKS at the LEC multicellular junctions and its association with the RAB6+ pool of CCL21ΔC-mCherry. Conclusively, our results showed that RAB6+ CCL21ΔC-mCherry containing vesicles require ELKS for exocytosis at the LEC multicellular junctions, which are the preferential sites of chemokine CCL21ΔC-mCherry exocytosis and DC transmigration.

8.9 Knockdown of RAB6 or its docking factor ELKS causes the accumulation of endogenous chemokine CCL2

To ascertain if siRNA mediated knock down of RAB6 or its docking factor ELKS also caused the accumulation of endogenous chemokine, I transfected cultured human primary dermal LECs with siRAB6, siELKS or siControl. I then incubated the monolayer with TNF-α for 24 h prior to fixation, to induce the expression of CCL2. After fixation, the cells were stained for CCL2. My results showed that siELKS treatment caused a build-up of chemokine CCL2, robustly at the LEC multicellular junctions, and to some extent also in the cell body (Fig. 6G-J in I). However, siRAB6 treatment did not cause the accumulation of CCL2 at the multicellular junctions, rather the CCL2 intensity in the whole LEC body was elevated (Fig. 6G-J in I). It is known that RAB6+ vesicles are transported via the microtubules, by attaching to the microtubule-associated motor protein, kinesin-1 (Grigoriev et al., 2007). It is possible, that the knockdown of RAB6 impaired the recruitment of kinesin, as reported in HeLa cells (Grigoriev et al., 2007). The disruption of this interaction could drastically slow-down the vesicle delivery process (Lee et al., 2015), which could hinder the arrival of vesicles at the LEC multicellular junctions. However, upon the siRNA mediated inhibition of ELKS, the interaction between RAB6 and kinesin-1 is not affected and therefore, the vesicles arrive normally at the LEC multicellular junction. Their accumulation, however, is caused because ELKS, which is required for the docking and fusion of RAB6+ vesicles, is not present to assist the exocytosis process (as shown in Fig. 5D in I, see section 8.8). Conversely, we found that upon siRNA mediated knockdown of RAB6, the CCL21ΔC-mCherry profusely accumulated at the

multicellular junctions (Fig. 6C-D in I), unlike the endogenous CCL2 (Fig. 6G-H in I). This could occur because CCL21ΔC-mCherry was overexpressed for a couple of days prior to fixation, and therefore a much greater number of vesicles were available for transport, leading to a higher build-up. Whereas, in the case of endogenous CCL2, the TNF treatment was done for only 24 h prior to fixation. Therefore, to reach the same level of accumulation, more time must be allowed.

8.10 RAB8A is necessary for the secretion of chemokine CCL21 and the dependent DC transmigration

Next, we focused our attention to RAB8, which together with ELKS and MICAL3 (mono-oxygenase), has been previously been indicated to be essential in the docking and fusion of RAB6+ vesicles (Grigoriev et al., 2011). My results (section 8.2 above) showed some colocalization of CCL21ΔC-mCherry with EGFP-RAB8A in high intensity vesicles present at the LEC multicellular junctions (Fig. EV2A and H-K in I). To check the relevance of RAB8A in the context of CCL21ΔC-mCherry+ vesicle docking and exocytosis, I decided to inhibit RAB8 function by using the expression of a dominant negative mutant of RAB8 in cells. A previous study had shown that the expression of RAB8A-T22N in cells caused the ablation of the exocytosis of RAB6+ vesicles (Grigoriev et al., 2011). RAB-GTPases are activated via the conversion from the inactive GDP bound state to GTP bound confirmation (Pfeffer, 2001; Segev, 2001; Grosshans et al., 2006). This conversion is facilitated by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) (Soldati et al., 1994; Ullrich et al., 1994; Li & Marlin, 2015). The dominant negative mutants of RAB-GTPases have higher affinity for binding with GDP and are therefore inactive (Peranen et al., 1996). They work by competing with the endogenous wildtype RAB-GTPases for binding with their regulatory proteins for example the GEFs (Hattula et al., 2002; Caviglia et al., 2019). This reduces the amount of GTP-bound wildtype RABs and hinders their function (Chen et al., 2003). To that end I sub-cloned a fluorescently tagged dominant negative mutant of RAB8A (EGFP-RAB8A-T22N), into a lentivirus vector (Hattula et al., 2002).

Next, to ascertain if EGFP-RAB8A-T22N impaired the secretion of CCL21 at LEC multicellular junctions, I carried out lentiviral co-transduction of cultured LEC monolayer with either EGFP (as a control) or EGFP-RAB8A-T22N and CCL21ΔC-mCherry. Using live fluorescent microscopy, I observed a drastic drop in the extent of CCL21ΔC-mCherry exocytosis, at the LEC multicellular junctions (Fig. 7A in I). Additionally, I collected the media from top of cultured LECs, and measured the fluorescence intensity of mCherry, using a spectrophotometer. I found that as compared to the EGFP control, the EGFP-RAB8A-T22N treated cells showed 40% drop in the fluorescence signal present in the culture media (Fig. 7B in I).

Furthermore, we observed that the overexpression of EGFP-RAB8A-T22N caused CCL21ΔC-mCherry to be profusely accumulated specifically at the LEC multicellular junctions and also in the LEC body (Fig. 7C-F in I). Moreover, this was not seen in cells overexpressing CCL21ΔC-mCherry and co-transduced with either EGFP or EGFP-RAB8A wildtype (Fig. 7C-F in I).

To validate that the inhibition of RAB8 caused the accumulation of, specifically, the RAB6+ pool of CCL21ΔC-mCherry vesicles, I stained the LEC monolayer overexpressing CCL21ΔC-mCherry and either of EGFP or EGFP-RAB8A-T22N with RAB6 antibody. I found that the accumulated CCL21ΔC-mCherry+ vesicles were also positive for RAB6 (Fig. 7G in I). This showed the dependence of peripheral CCL21ΔC-mCherry+ vesicles on RAB8 for their exocytosis and thereby supported the finding where RAB8 is required for the docking and exocytosis of RAB6+ vesicles (Grigoriev et al., 2011).

To test if RAB8A was also relevant for endogenous chemokine CCL2 delivery in LECs, we stained CCL2 in LEC monolayer overexpressing either EGFP, EGFP-RAB8A-T22N or EGFP-RAB8A wildtype and treated with TNF-α for 24 h prior to fixation. Similar to the accumulation of CCL21ΔC-mCherry, we observed strong build-up of CCL2 at LEC multicellular junctions in the samples transduced with EGFP-RAB8A-T22N, while neither the EGFP nor EGFP-RAB8A wildtype showed any significant effect (Fig. 7H-K in I).

Based on these results, we further tested if inhibiting RAB8A affected the transmigration of DCs across the LEC monolayer. To that end, we loaded LPS activated DCs on cultured LECs, co-transduced with either of CCL21ΔC-mCherry or CCL21-mCherry and EGFP-RAB8A-T22N or EGFP-RAB8A wildtype. With the help of fluorescent live microscopy, we saw 38% drop in DC transmigration events in samples expressing CCL21ΔC-mCherry/EGFP-RAB8A-T22N and about 14% drop in CCL21-mCherry/EGFP-RAB8A-T22N expressing LECs (Fig. 7M and movie EV13 in I). Therefore, our results confirmed that RAB8 is essential for the secretion of CCL21 at the LEC multicellular junction, and thereby also for DC transmigration.

To summarize, my thesis work resulted in the identification of previously unknown mechanism of leukocyte guidance to the site of transmigration. Previous studies had shown that leukocytes extravasate preferentially across the tri-cellular junctions of the BECs (Burns et al., 1997; Gorina et al., 2014). The BEC tri-cellular junctions were shown to express higher amount of ICAM-1, which was proposed to mark these sites for preferential transmigration by the leukocytes (Sumagin & Sarelius, 2010), However, it was not known how the leukocytes first arrived at these sites. On the other hand, in the context of the intravasation of leukocytes into the lymphatic vessels across the lymphatic endothelia, it was not known how the leukocytes choose the

transmigration sites. The results from my thesis provide the first evidence that the lymphatic endothelium can spatially target the release of chemokine, which directs the DCs to the LEC multicellular junctions where it drives the transmigration. My results show that the DCs sense the spatially targeted secretion of chemokine CCL21 at the LEC multicellular junctions. The exocytosed CCL21 possibly forms a gradient at the plane of the lymphatic endothelium which guides the DCs to the site of preferential transmigration.

8.11 Limitations of the study

(1) Mouse bone-marrow derived DCs (BMDCs) were employed to carry out the DC transmigration experiments, discussed in this thesis. The BMDCs are readily used in research because they are easy to procure in vitro, however, they might not fully recapitulate the tissue resident DCs (TRDCs). This is because the tissue microenvironment where the TRDCs thrive might shape their functional specialization (Chen et al., 2016). This might directly affect their antigen capture and presentation capability (Chen et al., 2016). BMDCs therefore cannot exactly mimic this in our cell culture model.

(2) One major limitation of this study is the need of overexpression of chemokine CCL21 in cultured primary human dermal LECs. This was essential since cultured LECs downregulate endogenous CCL21 (Wick, 2007). However, we used staining of endogenous CCL21 in vivo and endogenous CCL2 in cell culture to verify the identified secretory routes.

(3) Our transmigration model employed cultured primary human dermal LECs, overexpressing mouse CCL21-mCherry or CCL21ΔC-mCherry, and mouse DCs. However, ideally human derived DCs and human CCL21 should be employed in human LECs to alleviate specie dependent differences which might directly affect the transmigration efficiency.

(4) Our primary human dermal LEC culture model more closely represents the zipper junctions of pre-collector LECs in homeostasis and the lymphatic capillary LECs upon inflammation (Yao et al., 2012). Our explant results show that DCs preferentially transmigrate through the multicellular junctions of pre-collector LECs, in steady state. Since, the lymphatic capillaries are the major site of DC transmigration in vivo (Pflicke & Sixt, 2009), it should be tested if DCs also transmigrate across multicellular junctions of zippered lymphatic capillary endothelial cells upon inflammation in vivo/ex vivo. This was not addressed in this study and presents a limitation.

Conclusions and perspectives

In this study, using DC transmigration into the lymphatics as a model system, we have shown a novel cell biological concept where spatially targeted secretion of guidance cue is used to determine the site of cell migration. In doing so, this study provided the first proof of LEC multicellular junctions, as the "preferential sites" of leukocyte transmigration into the lymphatic vessel lumen. This finding was in-line with the already known transmigration of neutrophils across the BEC tri-cellular junctions (Burns et al., 1997; Wang et al., 2006; Gorina et al., 2014), and the integration of multiciliary cells into the lung epithelial multicellular junctions (Ventura et al., 2022).

Using extensive fast-live microscopy, I discovered that the RAB6+ pool of chemokine CCL21 is specifically exocytosed at the LEC multicellular junctions. This finding revealed, for the first time, how these junctions are prepared to guide the approaching DCs to their transmigration sites. I found that the RAB6+ vesicles containing the guidance cue (chemokine CCL21) are delivered to the LEC multicellular junctions – preferential sites of DC transmigration – via the microtubules, which are targeted to these sites. Accordingly, I found that the presentation of the guidance cue, at the LEC multicellular junctions, is essential for the transmigration of DCs. Obstructing the exocytosis of chemokine CCL21 at the LEC multicellular junctions indeed reduced DC transmigration across the LEC monolayer, in cell culture.

Conclusively, the novel findings of my thesis, showing the ways to control DC transmigration across the lymphatic endothelia, can now be tested *in vivo* and later also in pre-clinical disease models. Using this knowledge, translational therapies could be designed which might allow controlling antigen presentation and thus, the extent of T-cell activation and the resulting level of adaptive immune response in humans. Future therapies, developed based on this knowledge, would therefore help to resolve the complications associated with inflammation associated diseases such as transplant rejection, obesity, diabetes, cancer and rheumatoid arthritis (Dinarello, 2010; Bennett et al., 2018).

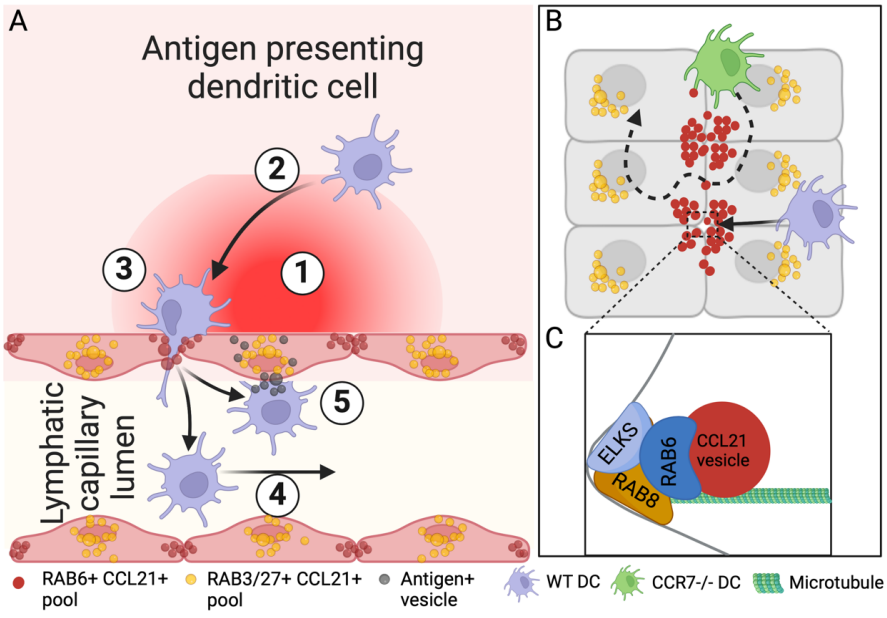

Figure 13 LEC multicellular junctions: the preferential sites of dendritic cell transmigration. The schematics (A-C) summarize the events related to DC transmigration into the lymphatic vessels. **(A)** First, (1) The lymphatic endothelial cells (LECs) of the initial lymphatics secrete chemokine CCL21, which forms a gradient in the surrounding interstitial space (Weber et al., 2013). (2) Antigen-presenting dendritic cells (DCs) sense chemokine CCL21 via their CCR7 receptor and are attracted towards the lymphatic capillaries (Forester et al., 1999; Ohl et al., 2004). (3) DCs approach the LEC surface and search for the transmigration site. Most of DC transmigration occurs at the LEC multicellular junctions (this thesis). (4) After locating the transmigration site, DCs enter the lymphatic vessel lumen (Russo et al., 2016). (5) Inside the lymphatic vessel lumen, the DC attaches to the luminal surface of the LECs. This interaction might allow an opportunity for the exchange of antigens between the LECs and DCs. It would be interesting to know, which pool of CCL21 mediates these interactions. Additionally, it is possible that besides chemokine CCL21, other proteins are also stored and transported in the same vesicles, which might assist in the transmigration of DCs. Future experimentation is needed to ascertain this. **(B)** DCs detect the LEC multicellular junctions by sensing the chemokine CCL21 at LEC multicellular junctions (this thesis). In the absence of CCL21/CCR7 signaling the DCs are not able to find the transmigration sites (this thesis). **(C)** The LEC multicellular junctions are distinct from the rest of LEC surface and junctions because of the enrichment of the RAB6+ CCL21+ vesicles, delivered by the microtubules which are targeted to these sites (this thesis). Moreover, the LEC multicellular junctions possess the RAB6-interacting proteins ELKS and RAB8 (this thesis), which are needed for the exocytosis of RAB6+ CCL21+ vesicles (Fourriere et al., 2019; Grigoriev et al., 2007; Nyitrai et al., 2020). This schematic was modified from (Liaqat et al., 2024). The schematic was created using BioRender.com.

My unpublished results (Figure 11), show that besides chemokines CCL21 and CCL2, many other chemokines are also expressed in LECs. It would now be interesting to study if the secretory mechanisms identified in this thesis are also relevant for the spatially targeted secretion of other chemokines. Also, it is possible that other chemokines, besides CCL21, could also take part in the guidance of DCs. It would be very interesting to find if this occurs, as it would open new ways to control the guided migration of DCs. It is known that in the blood endothelial cells, the RAB3/RAB27A+ Weibel-Palade bodies, carry many proteins including chemoattracts and adhesion molecule P-selectin, which are secreted upon vascular injury, and are responsible to attract the leukocytes to the wound site (Lowenstein et al., 2005). It is possible that in LECs too, the RAB6+ and RAB3D/RAB27A+ vesicles, besides storing chemokine CCL21, also contain other proteins which assist DC transmigration. Therefore, identification of the proteins, present in the LEC RAB6+ and RAB3D/RAB27A+ vesicles, could provide further insight into how the DC transmigration is orchestrated and could show additional ways to control this process.

My thesis primarily focuses on the ways to control the exocytosis of the RAB6+ pool of CCL21, due to its direct involvement at the site of preferential DC transmigration – the LEC multicellular junctions. However, CCL21 is also present in the RAB3/27A+ pool. It would now be interesting to find out the ways to control the exocytosis of CCL21 from the RAB3/27A+ pool. One way this could be achieved is by manipulating the regulatory proteins which control RAB-GTPases (Caviglia et al., 2019). Many effector proteins have been reported to associate with RAB27 and RAB3. These effectors include Slp4-a and Munc13-2, which interact with both RAB27A and RAB3, and Munc13-4 and MyRIP which interact with RAB27A (Nightingale et al., 2009; Rojo Pulido et al., 2011; Bierings et al., 2012; Zografou et al., 2012; van Breevoort et al., 2014; Conte et al., 2016; Chehab et al., 2017; Holthenrich et al., 2019; Nass et al., 2021). It is possible that that these effectors can also mediate the exocytosis of CCL21, and other cargo present in the RAB3/27A+ vesicles in LECs. Manipulation of these proteins could provide the tools to possibly affect the secretion of CCL21 from the RAB3/27A+ vesicles and to study the role of the RAB3D/RAB27A+ pool of chemokine CCL21, in the guidance and transmigration of DCs.

Apart from the transmigration of DCs across the lymphatic endothelia, other interactions between the LECs and DCs also occur. It is known that antigen exchange between the migrating DCs and LECs can take place (Kedl et al., 2017). LECs capture and archive the antigens upon incurring viral infection or vaccination (Tamburini et al., 2014). Previous reports have indicated that in tolerance, LECs can directly present antigens to cytotoxic T-cells (Cousin et al., 2021; Gkountidi et al., 2021). It would be interesting to study if LEC secretions guide the DCs to enable the exchange of antigens. This knowledge would provide further targets enhance adaptive immunity. Altogether,

the findings presented in this thesis and the future studies would be very essential in understanding how non-immune cells support immunity.

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