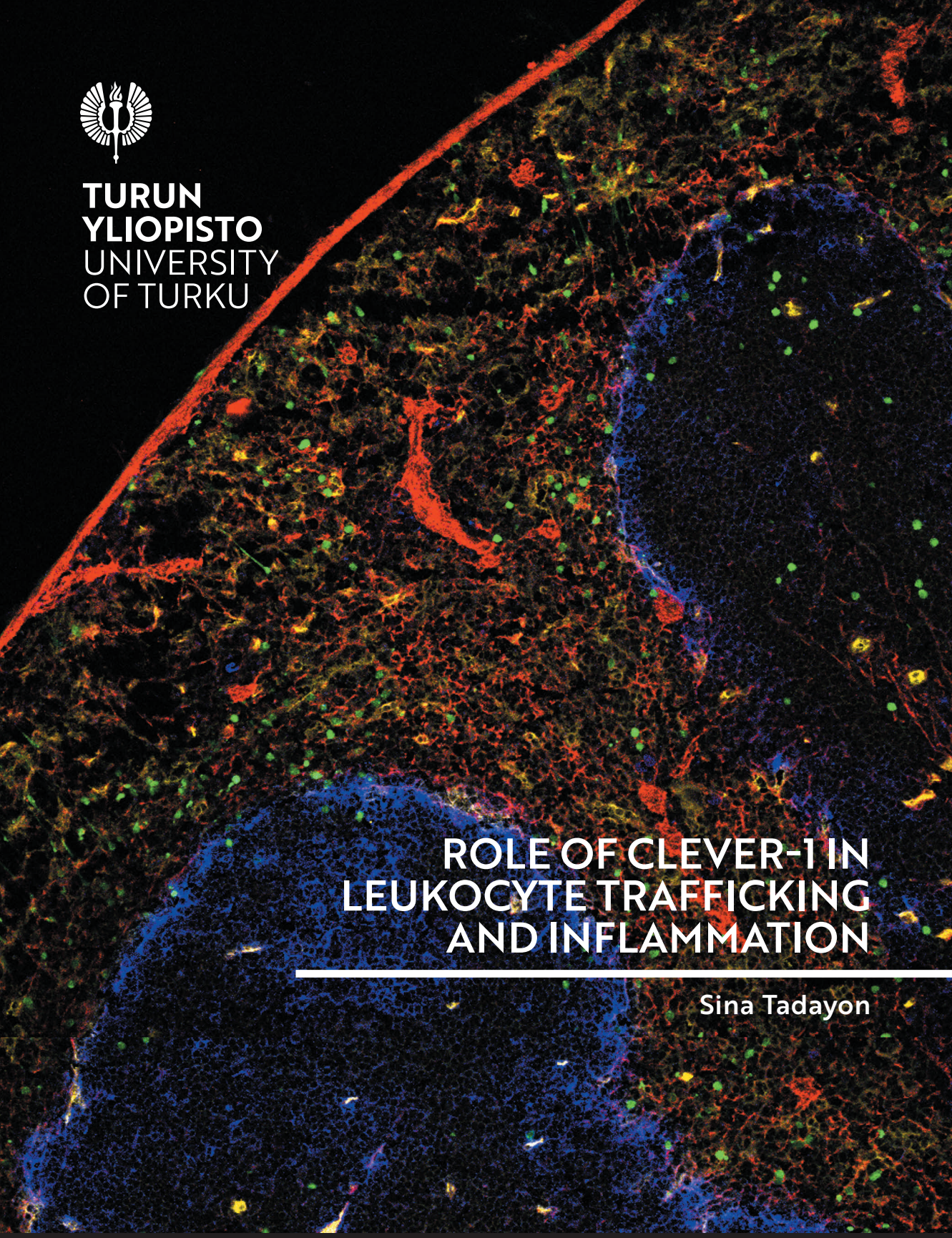

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*To everyone who helped me*

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## ABSTRACT

Secondary lymphoid organs, including lymph nodes (LNs) and the spleen, play an important role in protecting us against invading pathogens. While LNs are connected to lymphatic vessels and receive antigens from the peripheral tissues, the spleen is connected to blood vessels and filters blood-borne antigens. Migration of antigen presenting cells, such as dendritic cells (DCs), and lymphocytes into secondary lymphoid organs is crucial for initiating a proper adaptive immune response. Common lymphatic endothelial and vascular endothelial receptor-1 (Clever-1) is a multifunctional scavenger and adhesion receptor expressed on a subset of macrophages as well as endothelial cells and has been shown to selectively regulate leukocyte migration into different tissues. The role of Clever-1 in lymphocyte and dendritic cell migration into secondary lymphoid organs has not been thoroughly investigated. My doctoral studies describe new roles for Clever-1 in leukocyte trafficking and immune response.

In this thesis work, we showed that lymphocyte entry into the spleen is not a passive process forced by blood flow, but rather an active process regulated by Clever-1. We also discovered that lymphocytes preferentially enter the spleen via the vessels in the red pulp (RP) rather than marginal zone (MZ) sinuses or the white pulp (WP) vessels. These findings change the long-lasting dogma since 1973, which stated that lymphocytes enter the spleen via the MZ sinuses. We also identified Clever-1 as the first molecule that regulates lymphocyte migration into the spleen via the vasculature in the red pulp. In addition, we discovered that Clever-1 is expressed on peripheral lymphatic vessels and its absence reduces DC transmigration. Moreover, Clever-1, expressed on lymphatic endothelial cells (LECs), down-modulates DC activation and subsequently regulates the magnitude of antigen-specific T-cell responses in lymph nodes. In summary, this thesis work identified Clever-1 as a unique molecule that controls the migration of lymphocytes and DCs into secondary lymphoid organs, along with its crucial role in regulating antigen-specific immune responses.

KEYWORDS: Clever-1, lymph node, spleen, endothelial cells, lymphocyte migration, dendritic cell migration, antigen-specific immune response

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## TIIVISTELMÄ

Toissijaiset imukudokset mukaan lukien imusolmukkeet ja perna suojelevat meitä taudinaiheuttajia vastaan. Imusolmukkeet ovat suoraan kytköksissä imusuoniin ja vastaanottavat antigeenejä ympäröivistä kudoksista. Perna puolestaan on yhteydessä verisuoniin ja suodattaa veren kuljettamia antigeenejä. Antigeenejä esittelevien solujen kuten dendriittisolujen (DC) ja lymfosyyttien migraatio toissijaisiin imukudoksiin on elintärkeää toimivan hankitun puolustusvasteen käynnistämiseksi. Clever-1 on monitoiminnallinen reseptori, joka sitoo ja poistaa elimistön ei-toivottuja molekyylejä ja jota ilmennetään endoteelisoluissa. Clever-1:n on osoitettu valikoidusti säätelevän leukosyyttien migraatiota eri kudoksiin. Kuitenkin Clever-1:n toimintaa lymfosyyttien ja dendriittisolujen migraatiossa ei ole riittävästi tutkittu. Väitöskirjatyössäni kuvaan Clever-1:n ennen tuntematonta toimintaa leukosyyttien liikehdinnässä ja puolustusvasteessa.

Tässä väitöstyössä osoitin, että lymfosyytit eivät päädy pernaan vapaasti veren virtauksen voimalla vaan Clever-1 säätelee lymfosyyttien pääsyä pernaan. Havaitsin myös, että lymfosyytit kulkevat ensisijaisesti pernaan punaisen ytimen verisuonten kautta eivätkä valkoisen ytimen verisuonten tai marginaalialueen onteloiden kautta. Nämä löydökset kumoavat vuodelta 1973 lähtöisin olevan vallitsevan teorian, jonka mukaan lymfosyytit kulkevat pernaan marginaalialueen onteloiden kautta. Tutkimustulosteni perusteella Clever-1 on ensimmäinen tunnistettu molekyyli, joka säätelee lymfosyyttien siirtymistä pernaan. Tämän lisäksi havaitsin, että Clever-1:tä ilmennetään perifeerisissä imusuonissa, joissa se ohjaa dendriittisolujen trans-migraatiota kudoksista endoteelisolujen läpi imusuonien onteloon. Ilmentyneenä endoteelisoluissa Clever-1 vähentää dendriittisolujen aktivaatiota ja sitä kautta säätelee T-soluriippuvaisten antigeenivasteiden suuruutta. Kokonaisuutena väitöskirjatyöni osoittaa Clever-1:n ainutlaatuisuuden lymfosyyttien ja dendriittisolujen muuttoliikkeen/migraation sekä antigeenispefisisten puolustusvasteiden säätelijänä.

AVAINSANAT: Clever-1, imusolmuke, perna, endoteelisolut, lymfosyyttien migraatio, dendriittisolujen migraatio, antigeenispesifinen puolustusvaste

# Table of Contents

# Abbreviations

| | |
|---|---|
| Ac-LDL | Acetylated low-density lipoprotein |
| ACKR4 | Atypical chemokine receptor 4 |
| AGE | Advanced glycation end-product |
| APC | Antigen presenting cell |
| BC | Bridging channel |
| BCR | B-cell receptor |
| BEC | Blood endothelial cell |
| BMDC | Bone marrow derived-DC |
| BSA | Bovine serum albumin |
| C-LEC | Cortical LEC |
| CCL | Chemokine (C-C motif) ligand |
| CCR | C-C chemokine receptor |
| cDC | Conventional or classical DC |
| CDP | Common dendritic cells progenitor |
| CEACAM1 | Carcinoembryonic antigen-related cell adhesion molecule 1 |
| CHS | Contact hypersensitivity |
| CIITA | Class II transactivator |
| CLEC-2 | C-type lectin-like receptor 2 |
| Clever-1 | Common lymphatic endothelial and vascular endothelial receptor-1 |
| CLR | C-type lectin receptor |
| cMoP | Common monocyte progenitor |
| CMP | Common myeloid progenitor Cell |
| CR | Complement receptor |
| CTL | Cytotoxic effector $CD8^+$ T-cell |
| CTLA-4 | Cytotoxic T-lymphocyte antigen-4 |
| CX3CL1 | C-X3-C motif chemokine ligand 1 |
| CX3CR1 | C-X3-C chemokine receptor 1 |
| CXCL12 | C-X-C motif chemokine ligand 12 |
| CXCL13 | C-X-C motif chemokine ligand 13 |
| CXCR | C-X-C chemokine receptor type |
| CXCR4 | C-X-C chemokine receptor type 4 |

| | |
|---|---|
| DC | Dendritic cell |
| DC-SIGN | Dendritic cell-specific intercellular adhesion molecule-3-Grabbing Non-integrin |
| dLN | Draining lymph node |
| DN-DC | Double negative DC |
| DsRed | Red fluorescent protein |
| EGF | Epidermal growth factor |
| EpCAM | Epithelial cell adhesion molecule |
| Fas-1 | Fasciclin |
| FDC | Follicular dendritic cell |
| FITC | Fluorescein isothiocyanate |
| *FLT4* | *Fms-related tyrosine kinase 4* |
| FO B-cell | Follicular B-cell |
| Foxp3 | Forkhead P3 |
| FRC | Fibroblastic reticular cell |
| GlyCAM-1 | Glycosylation-dependent cell adhesion molecule-1 |
| GMP | Granulocyte-macrophage progenitor |
| HA | Hyaluronan |
| HDLEC | Human dermal lymphatic endothelial cells |
| HEV | High endothelial venule |
| HSC | Hematopoietic stem cell |
| HSEC | Hepatic sinusoidal endothelial cell |
| i.v. | Intravenously |
| ICAM | Intercellular adhesion molecule |
| iDC | Inflammatory DC |
| IES | Interendothelial slits |
| *Ifi202b* | *Interferon-inducible gene 202B* |
| IFN | Interferon |
| IL | Interleukin |
| IL-7Rα | IL-7 receptor α-chain |
| iNOS | Inducible nitric oxide synthase |
| intDC | Interstitial/dermal DC |
| IPA | Ingenuity Pathway Analysis |
| *Irak4* | *Interleukin-1 receptor-associated kinase 4* |
| Itgα7 | Integrin-α 7 subunit |
| KO | Knockout |
| LC | Langerhans cell |
| LEC | Lymphatic endothelial cell |
| LFA-1 | Lymphocyte function-associated antigen 1 |
| LN | lymph node |

| | |
|---|---|
| LNSC | Lymph node stromal cell |
| LPS | Lipopolysaccharide |
| LTβR | Lymphotoxin-β receptor |
| LYVE-1 | Lymphatic vessel endothelial hyaluronan receptor-1 |
| M-LEC | Medullary LEC |
| M2 | Type II macrophage |
| MAC-1 | Macrophage-1 antigen or αM-β2 integrin |
| MACRO | Macrophage receptor with collagenous structure |
| MAdCAM-1 | Mucosal vascular addressin cell adhesion molecule 1 |
| mDC | Myeloid DC |
| MDP | Macrophage-dendritic progenitor |
| MECA-79 | Peripheral node addressin antibody |
| medRC | Medullary FRC |
| MHC | Major histocompatibility complex |
| MHCI | Major histocompatibility complex class I |
| MHCII | Major histocompatibility complex class II |
| MLR | Mixed leukocyte reaction |
| MMM | Marginal zone metallophilic macrophage |
| moDC | Monocyte-derived DC |
| MRC | Marginal reticular cell |
| MZ | Marginal zone |
| MZ-BC | Marginal zone bridging channel |
| MZB | Marginal zone B-cell |
| MZM | Marginal zone macrophage |
| NLR | Nod-like receptor |
| NRP1 | Plexin-A1–neuropilin 1 |
| OVA | Ovalbumin |
| PALS | Periarterial lymphatic sheath |
| PAMP | Pathogen-associated molecular pattern |
| PBS | Phosphate-buffered saline |
| PD-1 | Programmed cell death protein 1 |
| PD-L1 | Programmed death-ligand 1 |
| pDC | Plasmacytoid DC |
| PECAM-1 | Platelet endothelial cell adhesion molecule |
| Pericyte | Myofibroblastic pericytic cell |
| PLVAP | Plasmalemmal vesicle-associated protein |
| Poly(I:C) | Polyinosinic-polycytidylic acid |
| PROX-1 | Prospero homeobox protein 1 |
| PRR | Pattern recognition receptor |
| PTA | Peripheral tissue antigen |

| | |
|---|---|
| qPCR | Quantitative polymerase chain reaction |
| RA | Retinoic acid |
| RANKL | Receptor activator of nuclear factor kappa-β ligand |
| RBC | Red blood cell |
| RP | Red pulp |
| RPM | Red pulp macrophage |
| S-LEC | Subcapsular sinus LEC |
| S1P | Sphingosine 1-phosphate |
| SCF | Stem cell factor |
| SCS | Subcapsular sinus |
| SI-CLP | Stabilin-1-interacting chitinase-like protein |
| SIGLEC-1 | Sialic acid binding Ig-like lectin 1 |
| SIGN-R1 | Specific intercellular adhesion molecule-3-grabbing nonintegrin-related 1 |
| SPARC | Secreted protein acidic and rich in cysteine |
| T-B border | T-cell and B-cell zone |
| T-cell | T-lymphocyte |
| TAM | Tumour-associated macrophage |
| TCR | T-cell receptor |
| $T_{FH}$ | Follicular helper T-cell |
| TGF-β | Transforming growth factor beta |
| TGN | Trans-Golgi network |
| $T_H$ | T helper type cell |
| TIP-DC | TNFα-iNOS-producing DC |
| TLR | Toll-like receptor |
| TNF | Tumour necrosis factor |
| TNFSF11 | Tumour necrosis factor ligand superfamily member 11 |
| TRC | T-cell zone fibroblastic reticular cell |
| $T_{reg}$ | Regulatory T-cell |
| TSLP | Thymic stromal-derived lymphopoietin |
| UPL | Universal ProbeLibrary |
| VCAM-1 | Vascular cell adhesion molecule 1 |
| VEGF | Vascular endothelial growth factor |
| VEGFR-3 | Vascular endothelial growth factor receptor-3 |
| WP | White pulp |
| αSMA | α-smooth muscle actin |

# List of Original Publications

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

I Tadayon S., Dunkel J., Takeda A., Halle O., Karikoski M., Gerke H., Rantakari P., Virtakoivu R., Pabst O., Salmi M., Hollmén M., and Jalkanen S., Clever-1 contributes to lymphocyte entry into the spleen via the red pulp, Science immunology, 2019; 4:33

II Tadayon S., Dunkel J., Takeda A., Eichin D., Virtakoivu R., Elima K., Jalkanen S. and Hollmén M., Lymphatic Endothelial Cell Activation and Dendritic Cell Transmigration Is Modified by Genetic Deletion of Clever-1, Frontiers in immunology, 2021; 12:61.

# 1 Introduction

Our body is constantly encountering different pathogens, and yet we rarely become sick. Why do we rarely get sick, or why do we develop a long-lasting immunity against some infectious diseases? Even though the answer to these questions is complicated, the simple answer would be that our body has a sophisticated defence mechanism that protects us against invading pathogens. Our immune system is equipped with an army of immune cells on 24-hours a day search-and-destroy missions throughout the body. These cells continuously shuttle between the blood and lymphoid organs, searching for antigens. The secondary lymphoid organs mainly consist of the spleen, lymph nodes (LNs), mucosal lymphoid tissues and Peyer's patches. These lymphoid organs provide a propagating centre, where the immune cells can encounter pathogens and become activated to eliminate the pathogens. The activation, type and magnitude of the immune response are ultimately defined by the molecular interaction between immune cells and non-hematopoietic cells. For an effective immune response, both the migration of immune cells and their molecular interaction must be tightly regulated. (K. Murphy & Weaver, 2016)

The location and structure of lymphoid tissues are one of the most compelling narratives in immunology. LNs are embedded in the network of lymphatic vessels that connect the LNs to the peripheral tissues and the bloodstream. Naïve lymphocytes migrate into LNs mostly through the blood vessels, while dendritic cells (DCs), a subpopulation of immune cells, and free antigens enter the LNs through the lymphatic vessels. The sinus area and lymphatic vessels of LNs are lined by lymphatic endothelial cells (LECs). LECs regulate the migration of immune cells into and out of LNs, but they also modulate the immune response. (Abbas et al., 2016)

Unlike the LNs, spleen is connected to the blood vasculature system filtering the blood-borne antigens and dead cells. The unique structure and location of the spleen enable this organ to actively trap and eliminate bloodborne antigens. In rodents, the spleen consists of white pulp (WP) and red pulp (RP), which are separated by the marginal zone (MZ). While immune cells constantly migrate to spleen, their migratory pattern has remained largely unknown. A long-lasting dogma since 1973

stated that immune cells enter the spleen via the MZ sinuses or the vasculature in the white pulp. (Mitchell, 1973)

Many adhesion molecules and their receptors regulate the migration of immune cells into the lymphoid tissues. The migration of immune cells and activation of adaptive immune response have been extensively studied, yet only a few molecules have been identified to regulate both the immune cells migration and the magnitude of the immune response. Common lymphatic endothelial and vascular endothelial receptor-1 (Clever-1) is an adhesion molecule and scavenger receptor expressed on inflamed blood endothelium and both afferent and efferent lymphatic vessels. Clever-1 has been shown to regulate the migration of lymphocytes into the LNs and inflamed tissues. In addition, Clever-1 is considered as an immunosuppressive molecule expressed on a subset of M2 macrophages. (Irjala, Johansson, et al., 2003; Karikoski et al., 2009; Viitala et al., 2019) My doctoral studies aimed to study the regulatory role of Clever-1 in the migration of immune cells into the lymphoid tissues as well as its role in regulating the antigen-specific immune response.

Genetic ablation of Clever-1 in mice, together with human and mouse *ex vivo* adhesion assays, showed that Clever-1 regulates the migration of $CD8^+$ T-cells and $B220^+$ B-cells into the spleen. In addition, our results showed that lymphocytes preferentially enter the spleen via the RP blood vessels rather than MZ sinuses or WP vessels. We also found that absence of Clever-1 downregulates the expression of chemokine (C-X-C motif) ligand 13 (CXCL13) on the spleen endothelium. CXCL13 has been shown to guide B-cells into the lymphoid organs, and its downregulation may also contribute to attenuated lymphocyte migration into the spleen. (Kanemitsu et al., 2005; Neely & Flajnik, 2015)

We also discovered that Clever-1 is constitutively expressed on the collecting and capillary lymphatic vessels and mediates DC transmigration from peripheral tissues into the lymphatic vessels. Despite a lower number of migratory DCs in the draining LNs (dLNs), KO mice were able to mount a more efficient $CD4^+$ T-cell proliferative response against ovalbumin. This could be explained by the higher expression of major histocompatibility complex class II (MHC II) molecules on DCs in the absence of Clever-1. Using genomic analysis and *in vitro* experiments, we showed that Clever-1, expressed on LECs, functions as an immunosuppressive molecule dampening the antigen-specific immune response. Therefore, even though a lower number of DCs can reach the dLN in the absence of Clever-1, they are more active and capable of mounting a stronger immune response. Taken together, these studies revealed a new function for Clever-1 in regulating the migration of immune cells into the lymphoid tissues as well as regulating the magnitude of the adaptive immune response, both of which are crucial for the function of the immune system.

# 2 Review of the Literature

## 2.1 Immune Cells and their roles in Immunity

The immune system consists of different components, including immune cells and lymphoid organs. The majority of immune cells, also known as leukocytes, arise from hematopoietic stem cells (HSCs) of the bone marrow and enter the bloodstream. After entering the bloodstream, they either enter peripheral tissues to search for pathogens or stay in blood circulation and enter the lymphatic system searching for pathogens to initiate the immune response. Either way, upon recognizing a pathogen, immune cells become activated to destroy the pathogen. Activated immune cells also secrete different cytokines, chemokines and other chemical mediators, which in turn induce a state of inflammation in the tissue to allow plasma proteins and other immune cells to enter the tissues. The initial immune response against a pathogen is initiated by innate immune responses, known as natural immunity. The innate immune response is the first line of defence against pathogens. Various cell types, including those of both hematopoietic and nonhematopoietic origin, are involved in an effective innate immune response. Hematopoietic cells involved in innate immunity consist of mast cells, basophils, eosinophils, natural killer cells and phagocytic cells such as DCs, monocytes, macrophages and neutrophils. Non-hematopoietic cells involved in innate immunity mainly consist of epithelial cells. (Turvey & Broide, 2010) The innate immune response is not specific to any particular antigen, but it is rapid and explosive once an infectious antigen invades the host. The innate immune response uses specific proteins, cell-associated receptors, and phagocytic cells to recognize and eliminate the antigens. Despite the effector functions of the innate immune response, it cannot completely eliminate the invading pathogens in many cases. Thus, a more effective and specific immune response is needed to eliminate the persistent pathogens. This specific immune response is called the adaptive immune response. (Medzhitov & Janeway, 1997a)

Adaptive immunity mainly consists of antigen-specific lymphocytes. Each mature lymphocyte has a unique variant of a prototype antigen receptor on its surface that enables the lymphocyte population to express an enormously variable repertoire of antigen receptors, which enable them to recognize and bind to a wide variety of antigens. There are two major types of lymphocytes responsible for two different types

of adaptive immune responses: 1) B-lymphocytes (B-cells) responsible for humoral immunity and 2) T-lymphocytes (T-cells) responsible for cell-mediated immunity. B-cells can recognize free antigens and become activated via their antigen receptors, B-cell receptors (BCRs), but most B-cells need help from activated helper T-cells for optimal activation. Upon activation, B-cells undergo clonal expansion and produce a large quantity of antibodies against their cognate antigen. Unlike B-cells, T-cells cannot be activated by direct exposure to antigens or by helper T-cells. Instead, T-cells are activated by DCs that express the antigen on their major histocompatibility complex (MHC) molecules, which is recognized by the antigen receptor, T-cell receptor (TCR), on T-cells. Upon activation, $CD8^+$ T-cells differentiate into cytotoxic T-cells capable of directly killing the infected cells. Alternatively, $CD4^+$ T-cells can differentiate into helper T-cells and secrete different cytokines to activate B-cells to produce antibodies. (Medzhitov & Janeway, 1997a; Paul, 2011)

An important subpopulation of leukocytes that link the innate and adaptive immunity is DCs. DCs constantly monitor the peripheral tissues searching for antigens. After encountering an antigen, they pick up the antigen, become activated and migrate via the afferent lymphatic vessels into the nearest dLNs, where they can present the antigen to T-cells and initiate the adaptive immune response (Figure 1). Lymphatic vessels are a pivotal component of the adaptive immune response, which link the infection site in peripheral tissues to the dLNs. Lymphatic vessels act as a highway to transfer the antigen-bearing migratory DCs and other immune cells to the dLNs, but also some free antigens are also transferred to the dLNs with lymph. (Alitalo, 2011) Since this thesis is about the migration and activation of immune cells involved in adaptive immune responses, I will discuss some of the significant components of the adaptive immune response in more detail in the following chapters.

### 2.1.1 T-Cells

T-cells are one of the major components of the adaptive immune response. T-cells constantly recirculate between the blood and secondary lymphoid organs. T-cells can either enter the dLNs by traversing through high endothelial venules (HEVs) or migrating via the lymphatic vessels from peripheral tissues. (Hunter et al., 2016) In the dLNs, T-cells migrate to the T-cell zone to meet antigen-bearing DCs and become activated (Figure 1). Depending on the nature and persistency of stimulus, they can further develop into either effector or central memory T cells. Effector T-cells can efficiently and quickly recognize peptide antigens on infected cells and respond to them. In fact, an effector T-cell's sensitivity to an antigen presented by the MHC complex increases by approximately 100 fold compared to naïve T-cells, yielding an efficient immune response against the presented antigen. (Hunter et al., 2016)

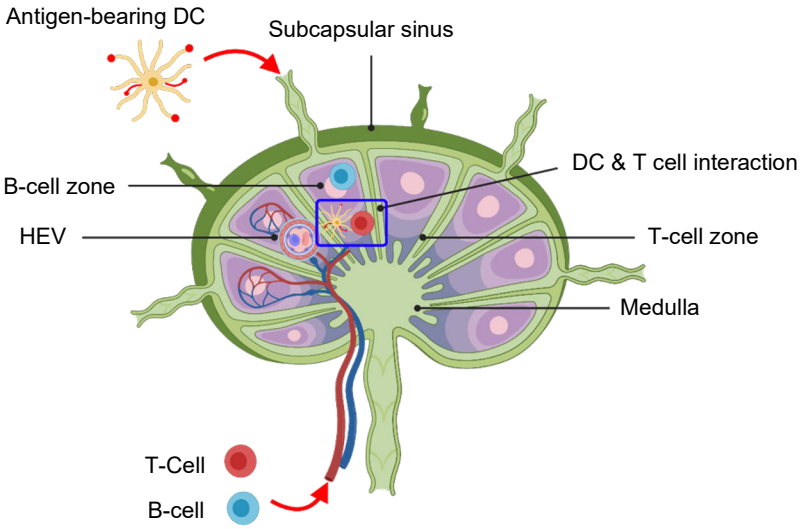


**Figure 1. A draining lymph node links the innate and adaptive immune response.** Dendritic cells (DCs) migrate to the draining lymph nodes (dLNs) via the afferent lymphatic vessels, whereas lymphocyte enter the dLNs via high endothelial venules (HEVs). Thereafter, T-cells migrate to T-cell zone, while B-cells home to B-cell zone. Antigen-bearing DCs interact with T-cells in the T-cell zone. Figure Modified from Murphy et al. (K. Murphy & Weaver, 2016) This figure was created with www.biorender.com.

T-cells can be categorized into two major categories, distinguished by their surface protein expression of CD4 and CD8. These surface proteins were initially used as surface markers for recognizing different T-cell functions. Later it was shown that their distinctions were due to their ability to recognize different classes of MHC molecules. While CD4 surface protein together with TCR bind to MHC II molecules on professional antigen presenting cells (APCs), CD8 surface protein together with TCR bind to major histocompatibility complex class I (MHC I) molecules on APCs and subsequently activate T-cells (Figure 2a and b). (K. Murphy & Weaver, 2016)

The interaction between TCR- and MHC complexes (Signal 1) in the presence of specific cytokines is crucial for the efficient activation and proliferation of T-cells. Nevertheless, it is not enough since an additional signal (Signal 2) is necessary to fully activate T-cells. The second signal is provided by the ligation of co-stimulatory molecules on APCs and their receptor on T-cells. Co-stimulatory receptors are transmembrane proteins that trigger intracellular signalling cascades through their cytoplasmic tails. Activation of the co-stimulatory molecules induces TCR signalling, leading to the complete activation of T-cells. (Li et al., 2009; K. Murphy & Weaver, 2016; Sharpe, 2009) Co-stimulatory molecules fall into three major families: 1) immunoglobulin superfamily (Ig), 2) Tumour necrosis factor (TNF) – TNF receptor (TNFR), and 3) emerging T-cell Ig and mucin (TIM) domain family.

(Li et al., 2009; Sharpe, 2009) Each T-cell subset expresses a particular type of co-stimulatory receptors and binds to specific co-stimulatory molecules on APCs (Table 1). (Carbone et al., 1997; Grewal & Flavell, 1998; Larsen & Pearson, 1997) The type of the co-stimulatory molecules has a crucial effect on the functional differentiation of effector T-cells. The co-stimulatory molecules can also differentiate the T-cell response either into immunosuppressive or pro-inflammatory subsets. In addition, the ligation of co-stimulatory molecules affects the activation state of other leukocytes. For example, ligation of CD40 and CD40L enhances DC maturation and their MHC expression, leading to upregulation of other co-stimulatory molecules, including CD80 and CD86. Ligation of CD40 also promotes the expression of inflammatory cytokines, such as TNF. (Ford & Larsen, 2009)

**Table 1.** Co-stimulatory molecules and their ligands.

| Co-stimulatory | Ligand | Cell Type expression |
|---|---|---|
| **CD27** | CD70 (Buchan et al., 2018) | Constitutive: Naïve T, B & NK cells<br>Inducible (CD70): T & B cells (Buchan et al., 2018) |
| **CD28** | B7-1 (CD80); B7-2 (CD86); (Bour-Jordan et al., 2011) | Constitutive: Naïve CD4 & CD8 T cell subsets (Bour-Jordan et al., 2011) |
| **CD30** | CD30L (K. W. Chan et al., 2002; Zeiser et al., 2007) | Constitutive: Tregs<br>Inducible: Activated effector/memory T-cells (K. W. Chan et al., 2002; Zeiser et al., 2007) |
| **CD40** | CD40L (Carbone et al., 1997) | Inducible: Activated T-cells, eosinophils, NK (Carbone et al., 1997) |
| **CD83** | CD154 (Wu et al., 2020) | Inducible: DCs (Lechmann et al., 2002; Prazma & Tedder, 2008) |
| **CTLA-4** | B7-1 (CD80)<br>B7-2 (CD86) (Bour-Jordan et al., 2011) | Constitutive: Tregs Inducible: Activated T-cells (Bour-Jordan et al., 2011) |
| **GITR** | GITRL (Shimizu et al., 2002; Watts, 2005) | Constitutive: Tregs (Shimizu et al., 2002)<br>Inducible: Activated effector T-cells, macrophages, NK& B-cells (Watts, 2005) |
| **ICOS** | ICOS-L2 | Inducible: T-cells (Hutloff et al., 1999; Wikenheiser & Stumhofer, 2016) |
| **OX40** | OX40L (Lane, 2000) | Inducible: activated T-cells (Buchan et al., 2018; Lane, 2000) neutrophils (Baumann et al., 2004) |
| **TIM-1** | TIM-1<br>TIM-4 (Freeman et al., 2010; Umetsu et al., 2005) | Inducible: Activated CD4 & CD8 T-cells (Umetsu et al., 2005) |
| **TIM-2** | Semaphorin4A (Kumanogoh et al., 2002)<br>H-ferritin (T. T. Chen et al., 2005) | Inducible: T-cells (Th2) & subset of B-cells (Chakravarti et al., 2005) |
| **TIM-3** | Galectin-9 (Zhu et al., 2005) | Constitutive: Tregs<br>Inducible:<br>Innate: NK cells, DCs & monocytes - humans; mast cells, macrophages & DCs – mice<br>Adaptive: subset activated CD4 & CD8 T-cells, terminally differentiated Th1 cells, Th17 cells (Boenisch et al., 2010) |

Upon activation, $CD8^+$ T-cells can differentiate into cytotoxic effector $CD8^+$ T-cells (CTL) and directly kill the cells that present the antigen peptide on their MHC I molecules. Unlike cytotoxic $CD8^+$ T-cells, activated $CD4^+$ T-cells cannot directly kill the infected cells, but instead they develop into different effector subsets called T helper cells ($T_H$) and depending on the nature of the immunological insult, these cells can secrete a wide range of cytokines and regulate the extent of the immune response and polarize the immune response into a pro-inflammatory or immunosuppressive response. (F. E. Lund & Randall, 2010; Opferman et al., 1999; Veiga-Fernandes et al., 2000) $T_H$ cells are also essential for activating other immune cells, such as B-cells, DCs and $CD8^+$ T cells. For instance, $T_H$ cells can secrete large quantities of interferons (IFN), which increases the expression of MHC molecules and enhances the antigen presentation function of MHC molecules, and consequently amplify the magnitude of the immune response against the antigens. (Veiga-Fernandes et al., 2000; Ye et al., 2018) $CD4^+$ effector T-cells consist of at least five subsets, including $T_H1$, $T_H2$, $T_H17$, follicular helper T cells ($T_{FH}$) and regulatory T-cells ($T_{reg}$). The first four subsets of $T_H$ cells contribute to the adaptive immune response by activating their target cells, while $T_{reg}$ cells control the extent of immune activation. (Josefowicz et al., 2012; K. Murphy & Weaver, 2016) $T_H1$ cells are critical for immunity against many intracellular pathogens, such as viruses and intracellular bacteria. $T_H1$ cells can be characterized by the expression of inflammatory cytokines, such as IFN-ɣ and interleukin (IL)-2. IL-2 is a crucial inflammatory cytokine that increases the survival of $CD8^+$ T-cells and downregulates apoptotic molecules. IL-2 also antagonizes IL-4 and subsequently inhibits $T_H2$ cell differentiation. (Boise et al., 1995; Larsen & Pearson, 1997; H. W. Lee et al., 2002; Rogers et al., 2001) IFN-ɣ is also an inflammatory cytokine that activates the immune response and increases DCs maturation. $T_H2$ cells are essential for immunity against extracellular parasites. (Xu et al., 2019) $T_H2$ cells can be distinguished by their production of IL-4, IL-5, IL-9, IL-10, IL-13 and IL-25. (Hohl, 2014) IL4, IL-5 and IL-13 are the most important signature cytokines of the $T_H2$ response. $T_H2$ cytokines stimulate B-cells to undergo isotype switching to produce IgE and IgG and activate eosinophils. (Hohl, 2014)

$T_{FH}$ is the main T-cell population for antibody responses. $T_{FH}$ cells can be distinguished from $T_H1$ and $T_H2$ cells by their expression of C-X-C chemokine receptor type 5 (CXCR5). Moreover, $T_{FH}$ cells express a large quantity of IL-21, which modulates isotype switching and Ig production by B-cells. (Pène et al., 2004) Another important subset of helper T-cells is $T_H17$ cells. $T_H17$ cells are characterized by the expression of IL-17, IL-17F, IL-21 and IL-22. The $T_H17$ response is crucial in immune response against the intracellular pathogens. On the other hand, inappropriate activating of $T_H17$ response is associated with many experimental autoimmune diseases. (Korn et al., 2009) Finally, unlike the function of other $T_H$

cells, $T_{regs}$ maintain tolerance by suppressing potentially harmful activities of effector T-cells. $T_{regs}$ can be characterized by the expression of Forkhead P3 (Foxp3) transcription factor, cytotoxic T-lymphocyte antigen-4 (CTLA-4), CD25, and transforming growth factor-beta (TGF-β). (W. J. Chen et al., 2003; Hori et al., 2017; Triplett et al., 2012; Walunas et al., 1994)

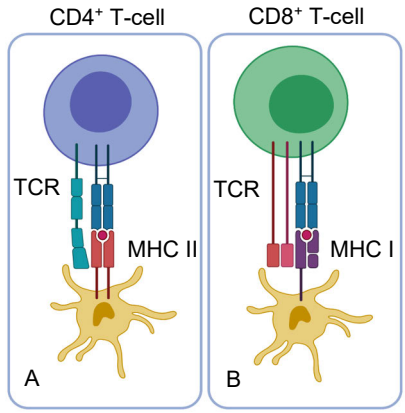


**Figure 2.** CD4 and CD8 surface proteins on T-cells bind to different classes of major histocompatibility complex (MHC) molecules on antigen presenting cells. A) CD4 together with T-cell receptor (TCR) bind to major histocompatibility complex class II (MHC II) molecules. B) CD8 together with TCR bind to major histocompatibility complex class I (MHC I) molecules. Figure Modified from Murphy et al. (K. Murphy & Weaver, 2016) This figure was created with www.biorender.com.

### 2.1.2 B-Cells

B-cells are another important component of adaptive immunity. B-cells have three subsets: (i) B-1 cells, which mainly originate from the fetal liver and are located in the pleural and peritoneal cavities. (ii) B-2 cells, also known as follicular B-cells (FO B-cells), which primarily originate from the bone marrow and are located in the B-cell zone of LNs, Peyer's patches, and spleen. (iii) Marginal zone B-cells (MZ B-cells), which have different origins and are mainly located in the marginal zone of the spleen. (Allman & Pillai, 2008) B-cells can also recognize and bind the antigens by their B-cell receptors (BCRs), a membrane form of an immunoglobulin. A secreted form of an immunoglobulin is called an antibody and is produced by terminally differentiated B-cells, known as plasma cells and plasmablasts. Unlike cytotoxic T-cells that can directly eliminate the invading pathogens, B-cells produce specific antibodies against the antigens of pathogens. Antibodies recognize these antigens to neutralize the invading pathogens or to recruit other immune cells to

destroy them. Antibodies are the main component of the humoral response of adaptive immunity.

Based on the location and the way through which B-cells are activated, B-cell activation can be divided into either T-dependent or T-independent. (Allman & Pillai, 2008) In a T-independent adaptive immune response, B-cells, particularly B-1 and MZ B-cells, directly respond to a free intact antigen, such as polysaccharides or lipidic antigens, and initiate the humoral immune response. (Zubler, 2001) Unlike the T-independent response, in which B-cells recognize an intact free antigen, in a T-dependent response, B-cells first recognize the antigen on follicular dendritic cells (FDCs) and $CD169^+$ macrophages. In this type of response, the presence of $T_{FH}$ cells is necessary for the full activation of B-cells. FO B-cells and MZ B-cells usually initiate the T-dependent response. (Batista et al., 2001; Hartley et al., 1991; Russell et al., 1991) In addition to FDCs and $CD169^+$ macrophages, recently, an elegant intravital microscopy study showed that a subset of conventional or classical DCs (cDCs), namely cDC2, located around HEVs, are able to activate newly-arrived B-cells in the extrafollicular region, suggesting an essential role for cDCs in priming B-cells. (Qi et al., 2006) Apart from the type antigen-presenting cell, many studies have shown that displaying antigens by antigen presenting cells can activate B-cells more efficiently than soluble antigens. (Batista et al., 2001; Hartley et al., 1991; Russell et al., 1991) One possible reason for this might be that B-cells constantly encounter a pool of soluble self-antigens. Thus, high reactivity to soluble antigens may increase the chance of autoimmunity.

Upon recognizing antigen, B-cells rapidly migrate and congregate in the border between the T-cell and B-cell zone (T-B border), where they can interact with pre-activated $T_{FH}$ cells for full activation. (Garside et al., 1998) The ligation of CD40 expressed on B-cells and CD40L expressed on $T_{FH}$ cells is crucial for fully activating the pre-$T_{FH}$ and B-cell response. The activated $T_{FH}$ cells produce different cytokines, including IL-4 and IL-21, resulting in the maturation, proliferation, and differentiation of B-cells. Activated B-cells can either form the germinal centre (GC) or migrate to the extrafollicular region and differentiate into plasmablasts and plasma cells. (T. D. Chan et al., 2009; Foy et al., 1994; Garside et al., 1998; Han et al., 1995; Jacob & Kelsoe, 1992; Kuppers et al., 1993; MacLennan et al., 2003) The migration of B-cells to the extrafollicular region or remaining to form the GC is highly dependent on the chemokine receptors expressed by B-cells, including CXCR5, C-X-C chemokine receptor type 4 (CXCR4) and C-C chemokine receptor type (CCR)-7, and their respective ligands, including chemokine (C-X-C motif) ligand 13 (CXCL13), C-X-C motif chemokine 12 (CXCL12) and chemokine (C-C motif) ligand (CCL)-19. Thereafter, plasmablasts can undergo rapid clonal expansion, resulting in the rapid generation of terminally differentiated short-lived plasma cells, which are the immediate source of antibody production. Alternatively, activated B-

cells can differentiate into long-lived memory B-cells, which form the basis for immune memory. (T. D. Chan et al., 2009; Cyster et al., 2000; Förster et al., 1996; Haynes et al., 2007; Reif et al., 2002)

### 2.1.3 Dendritic cells (DCs)

DCs are one the most critical components of the adaptive immune response. DCs connect the innate and adaptive immune responses by pinocytosing an antigen, processing it, and generating a peptide antigen to present it to naïve T-cells to initiate the adaptive immune response. DCs can be characterized by medium to high expression levels of MHC molecules and the absence of some of the lineage markers, such as CD3 (T-cells), CD14 (monocytes), CD56 (NK cells) and CD34 (stem cells). In addition, DCs also express integrin CD11c and different co-stimulatory molecules, including CD40, CD80, CD83 and CD86. (Teunissen et al., 2012) Originally, DCs were divided into lymphoid and myeloid groups, but this classification did not reflect their functionality. Thereafter, DCs were classified based on their functionality, but this classification, in turn, did not reflect their functional plasticity. Thus, recently, DCs were divided based on their ontology, which often relates to their functions. In this new classification system, DCs and their related myeloid lineages are divided into plasmacytoid DCs (pDCs), cDCs, Langerhans cells (LCs) and monocyte-derived DCs (moDCs) (Figure 3). (Blom et al., 2000; Chehimi et al., 1989; Hampton & Chtanova, 2019; Kashem et al., 2017) All of these subsets, with the exception of LCs, arise from hematopoietic stem cells in the bone marrow and are released into the blood circulation. However, after entering the blood circulation, their fate and developmental pattern are entirely different. In the following chapters, I briefly discuss the different populations of DCs.

#### 2.1.3.1 Different Subsets of DCs

##### 2.1.3.1.1 pDCs

pDCs, also known as natural IFN-α/β producing cells, originate from HSCs and enter the bloodstream in a fully developed form. (Chehimi et al., 1989) pDCs are rarely present in peripheral tissues at steady-state but have the ability to migrate into peripheral tissues upon inflammation. (Asselin-Paturel et al., 2003; Nakano et al., 2001) pDCs eventually leave the blood circulation and enter the lymphoid organs (Facchetti et al., 1988), such as tonsils (Grouard et al., 1997) and thymus (Figure 3). (Blom et al., 2000; Villadangos & Young, 2008) It has also been reported that pDCs may arise from lymphoid progenitors. (Rodrigues et al., 2018) pDCs can be

characterized by low expression levels of co-stimulatory and MHC II molecules at steady-state. pDCs can be identified by their expression of CD45R, CD45RA, CD317 in mice, and CD303, CD304, CD123 and CD45RA in humans. pDCs also have negative (human) or low (mouse) expression of integrin CD11c. (Mittag et al., 2011)

pDCs express a limited range of pattern recognition receptors (PRRs), including Toll-like receptor (TLR) 7 and 9 (Kadowaki, Ho, et al., 2001). Even though pDCs are poor antigen presenting cells for priming naïve T-cells, they play an essential role in fighting blood-borne pathogens. Upon encountering pathogens, such as viruses (Cella et al., 2000) and bacterial DNA (Kadowaki, Antonenko, et al., 2001), pDCs rapidly secrete a large volume of type I interferons, tumour necrosis factor (TNF), IL-6, and upregulate the expression of co-stimulatory molecules. (Reizis et al., 2011) These cytokines support the functions of other leukocytes, such as B-cells and NK cells, to fight the invading pathogens. Nonetheless, it has been shown that pDCs can also present the peptide antigen on their MHC I and MHC II molecules to activate T-cells. (Cella et al., 2000; Kadowaki, Antonenko, et al., 2001; Reizis et al., 2011)

#### 2.1.3.1.2 cDCs

In contrast to pDCs that enter the bloodstream fully developed, newly generated cDCs are considered immature. (Merad et al., 2013; Villadangos & Schnorrer, 2007; Villadangos & Young, 2008) Based on ontogeny, cDCs can be divided into two subsets: cDC1 and cDC2. The cDC1s express higher levels of IL-12 than cDC2s and uniquely express TLR3. (Crozat et al., 2010; Guilliams et al., 2014) The cDC1s mainly cross-present antigens to $CD8^+$ T-cells, whereas cDC2s presents antigens to $CD4^+$ T-cells. However, it has also been shown that cDC2s may be able to cross-present an antigen to $CD8^+$ T-cells to some extent. (Eisenbarth, 2019; Pooley et al., 2001) The cDC1 subset is a relatively rare population in both mouse and human dermis and can be characterized by the expression of CLEC9A (Zhang et al., 2012), chemokine receptor XCR1 (Dorner et al., 2009), IRF8, TLR3, CADM1, CD103, CD8α and DEC205. (Bursch et al., 2007; Malissen et al., 2014; T. Tamura et al., 2005) Since cDC1s in mouse dermis express high levels of CD103, this population is also known as $CD103^+$ dermal DCs. The mouse dermal cDC1s can also express langerin (CD207), a surface marker for LCs (Figure 3). (Bursch et al., 2007) Dermal cDC1s lack CD11b, CD172α , CD14, and C-X3-C chemokine receptor 1 (CX3CR1). (Geissmann et al., 2010) On the other hand, the cDC2s can be found in both lymphoid and non-lymphoid tissues, and in contrast to cDC1, they are the most abundant DC population in both mouse and human dermis. (Malissen et al., 2014) Dermal cDC2s can be characterized by their expression of CD11b, CD301b,

CX3CR1, DC immunoreceptor 2 (DCIR2), signal regulatory protein-α (SIRPα), IRF4, and CD4. (Satpathy et al., 2012; S. Suzuki et al., 2004; Williams et al., 2013; Yi & Cyster, 2013)

In addition to these subsets, cDCs can be ultimately subdivided into LN-resident and migratory DCs at their steady-state. After release into the bloodstream from the bone marrow, most cDCs migrate to peripheral tissues and dLNs, where they are called tissue-resident cDCs or LN-resident cDCs, respectively. However, a subset of cDCs will stay in the bloodstream and can be distinguished from pDCs through the expression of integrin CD11c. (Villadangos & Young, 2008) Tissue-resident cDCs, also known as dermal cDCs, spend their entire life span in this immature state unless they either encounter a pathogen or receive a maturation signal. Once activated, tissue-resident cDCs acquire migratory properties that enable them to leave the peripheral tissues and migrate into the nearest dLN, where they are called migratory cDCs (Figure 3). (Merad et al., 2013; Villadangos & Schnorrer, 2007; Villadangos & Young, 2008)

LN-resident cDCs migrate into the dLNs via the HEVs, where they stay their entire life span. Antigens can either arrive into the LNs via the afferent lymphatic vessels or be transferred by other immune cells. LN-resident cDCs can present the acquired antigens to both $CD4^+$ and $CD8^+$ T-cells. (Allan et al., 2006; Ersland et al., 2010; Gurevich et al., 2017) In fact, an elegant *in vivo* study showed that activation of cytotoxic T-cells requires antigen presentation by nonmigratory $CD8^+$ DCs rather than skin-derived migratory DCs. (Allan et al., 2006)

Despite their name, resident DCs are very motile within the LNs and usually survey different areas of LNs. (Allan et al., 2006; Ersland et al., 2010; Gurevich et al., 2017) The migratory DCs can be distinguished from the LN-resident DCs by their higher expression of MHC II and CD11c integrin at steady-state. (Ohl et al., 2004) However, depending on the type and extent of inflammation, upregulation of MHC II and CD11c on both migratory and LN-resident DCs make it challenging to distinguish them only based on these markers. (Eisenbarth, 2019; Merad et al., 2013; Waithman et al., 2013)

#### 2.1.3.1.3 moDCs

moDCs are a heterogeneous group of DCs, including inflammatory DCs (iDCs) and TNF/iNOs producing-DCs. moDCs rise from $Ly6C^{hi}$ monocytes and, like other DCs, are involved in clearing pathogens, presenting antigens to T-cells, and producing different cytokines. (Mildner et al., 2013; Segura & Amigorena, 2013) The moDCs are phenotypically similar to cDC2s and express MHC II, CD11c and CD11b (Plantinga et al., 2013), but due to their monocytic origin, they can be discerned by their expression of CD64 (Figure 3). (Tamoutounour et al., 2012) moDCs are rarely

present at steady-state but rapidly migrate into the inflamed tissue, indicating the role of inflammation in triggering their migration and differentiation. (Tamoutounour et al., 2013) Ly6C$^{hi}$ monocytes first enter the inflamed tissues, and while maturing and acquiring MHC class II expression, they lose their Ly6C expression. (Tamoutounour et al., 2013) Like cDCs, their migration into the inflamed tissues highly depends on CCR2. (Samstein et al., 2013) After capturing antigen in the peripheral tissues, moDCs can migrate into the dLNs and present the acquired antigen to T-cells. However, it has been shown that they are not as efficient as cDC2s in priming both CD4$^+$ and CD8$^+$ T-cells. (Tamoutounour et al., 2013)

#### 2.1.3.1.4 LCs

LCs are mononuclear phagocytes in the tissue that are traditionally classified with DCs. However, recently based on ontogeny they have been re-classified with macrophages. Like DCs, they can migrate into the dLNs at both steady-state and during inflammation and reach the deep T-cell zone to activate T-cells. LCs have an embryonic origin and can self-renew in situ (Figure 3). (Gerner et al., 2012; Kashem et al., 2017; Y. Wang et al., 2012) LCs can mainly be found in the interfollicular and follicular epithelium, where they can capture epidermal antigens.

LCs are equipped with long dendrites that enable them to protrude the tight junction of the outermost layer of the epidermis, known as the stratum corneum, to capture antigens. (Kubo et al., 2009) LCs can be characterized by their expression of MHC II, CD207, CD11b, XCR1 and epithelial cell adhesion molecule (EpCAM). EpCAM has been reported to decrease the LC-keratinocyte adhesion and promote the migration of LCs. (Gaiser et al., 2012)

#### 2.1.3.1.5 Double-negative DCs

Mouse skin and dLNs contain a minor population of migratory DCs, that lack XCR1 and CD207 and are referred to as double-negative dDCs (DN-DCs). These double negative dDCs express very low levels of CD11b, CD103 and CD326, but intermediate levels of CCR2 and CX3CR1 (Figure 3). They also express high levels of MHC II (Henri et al., 2010; Hohl et al., 2009; Mollah et al., 2014; Tamoutounour et al., 2013). Moreover, like other DC subsets, their migration to dLNs highly depends on their CCR7 expression. It has been shown that they are among the first migratory DCs that arrive in the dLN upon inflammation and reach the peak 24 to 48 hours after inflammation (Henri et al., 2010).

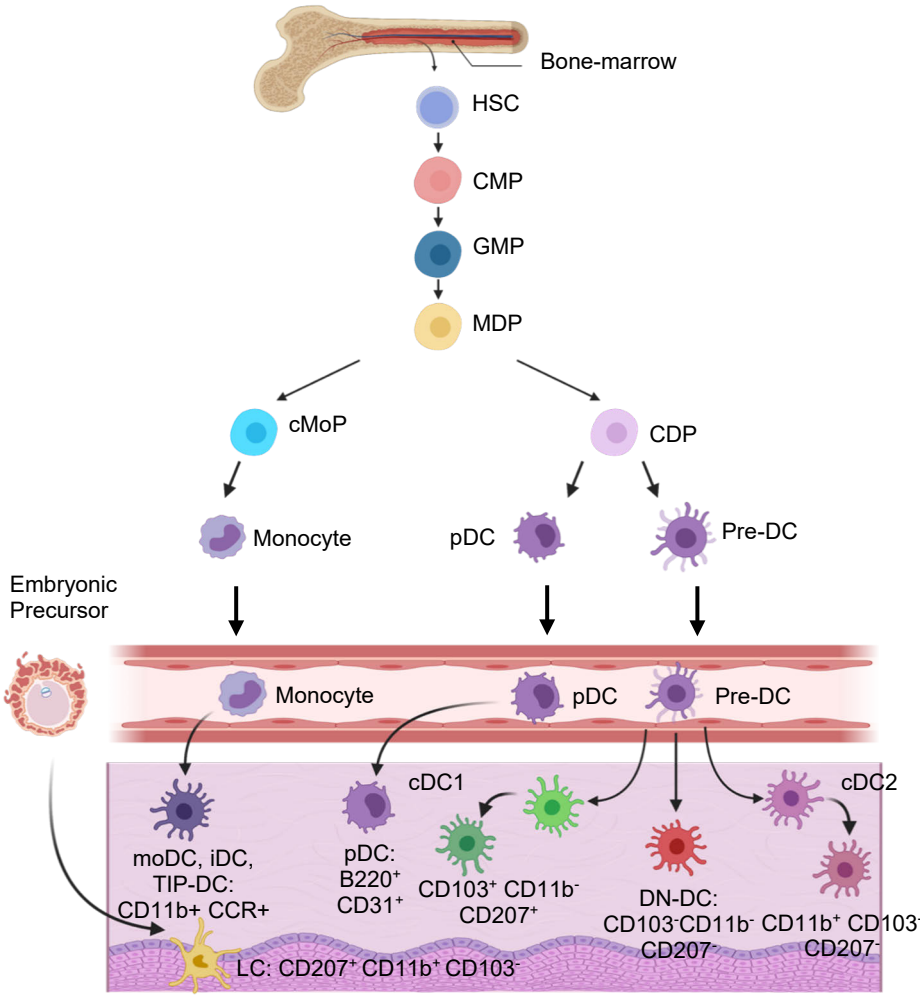


**Figure 3. DC subsets in mouse skin**. DCs in the skin have distinct origins. While monocytes, pre-DCs and plasmacytoid-DC (pDCs) originate from the hematopoietic stem cells (HSCs), Langerhans cells (LCs) have an embryonic precursor. The common myeloid progenitor cell (CMP) arises from the HSCs. Thereafter, the CMP differentiates into a granulocyte-macrophage progenitor (GMP) and further to macrophage-dendritic progenitor (MDP). The MDP can differentiate to either common dendritic cells progenitor (CDP) or common monocyte progenitor (cMoP). CDP can differentiate to pre-DC and pDC. Pre-DC can then differentiate into double negative DCs (DN-DC), conventional DCs(cDC)-1 and -2. cMoP can differentiate into monocytes. Upon insult, monocytes rapidly migrate to the site of inflammation and differentiate into monocyte-derived DCs (moDC), inflammatory DCs (iDC) and TNFα-iNOS-producing DCs (TIP-DC). Figure Modified from the following references: (Eisenbarth, 2019; Schlitzer & Ginhoux, 2014; Shortman & Liu, 2002) This figure was created with www.biorender.com.

#### 2.1.3.2 DC activation

Immature DCs are one of the most efficient immune cells in capturing antigens. Immature DCs are able to internalize a large volume of extracellular fluid via macropinocytosis, a mechanism by which they can take up and ingest the extracellular fluid. Moreover, they constitutively express high levels of endocytic receptors, including macrophage mannose receptor (MR or CD206), which enable them to capture and internalize antigens. (Sallusto et al., 1995) After being activated through recognition of pathogen structures by PRRs and endocytosing a pathogen, DCs undergo a complex process of maturation, during which DCs (i) change their morphology and lose their adhesive structure(Trombetta & Mellman, 2005); (ii) reduce their endocytic/phagocytic activities(Russo et al., 2013); (iii) upregulate genes involved in antigen presentation, such as MHC molecules, and translocate MHC class II compartment to the cell surface (Cella et al., 1997; Pierre et al., 1997); (iv) upregulate co-stimulatory molecules, such as CD80, CD86 and CD40 (Caux et al., 1994), and the production of cytokines, such as IL-12 (Heufler et al., 1996); and (v) change their migratory behaviour by upregulating the expression of CCR7 while downregulating inflammatory chemokine receptors. (Sallusto et al., 1998; Sozzani et al., 1998)

DCs express invariant innate PRRs, through which they detect a wide range of pathogen structures, such as those from viruses, bacteria, and damaged cells. PRRs recognize a molecular pattern on pathogens known as pathogen-associated molecular patterns (PAMPs). PAMPs are absent from the host's cells but are a part of many microorganisms. (Akira et al., 2006; Medzhitov & Janeway, 1997b; K. Murphy & Weaver, 2016) There are at least three PRR families of molecules involved in the recognition of PAMPs: (i) TLRs can recognize viral RNA, double stranded DNA and PAMPs derived from extracellular bacterial components; (ii) Nod-like receptors (NLRs) that detect intracellular bacterial invasion; and (iii) cell surface C-type lectin receptors (CLRs) that recognize and bind carbohydrate moieties of glycoproteins. (K. Murphy & Weaver, 2016)

##### 2.1.3.2.1 Toll-like Receptors

TLRs can be characterized based on the ligand that they recognize. For example, TLR1, TLR2, TLR4 and TLR6 recognize lipids, while TLR3, TLR7, TLR8 and TLR9 can detect nucleic acids. (Akira et al., 2006) Different subsets of DCs express a different subfamily of TLRs, which enables them to recognize different pathogens. For instance, in humans interstitial/dermal DCs (intDCs) express TLR2, TLR4, as well as TLR5 and can recognize bacterial PAMPs(Van der Aar et al., 2007), whereas myeloid DCs (mDCs) express TLR1 through TLR8 and TLR10, and can recognize a wider variety of pathogens, including viral dsRNA and bacterial PAMPs. (K.

Murphy & Weaver, 2016; Smed-Sörensen et al., 2012; Van der Aar et al., 2007) Notably, the ligation of different TLRs activates different molecular signals, consequently leading to differences in DC maturation, thereby initiating a distinct immune response profile. (Kapsenberg, 2003) For instance, lipopolysaccharide (LPS) of the outer membrane of *Escherichia coli* activates DCs through TLR4 and induces IL-12 (p70) secretion, thereby triggering a Th1 immune response, whereas, *Porphyromonas gingivalis* LPS triggers DCs through their TLR2, inducing IL-10 secretion and subsequently activating a Th2 response. (Pulendran et al., 2001) Similarly, peptidoglycan, an agonist for TLR2, triggers IL-10 expression by DCs, whereas Poly(I: C), a TLR3 ligand, triggers TNF-α expression. (Flacher et al., 2006)

#### 2.1.3.2.2 C-type Lectin Receptor (CLR)

Another PRR that DCs use to recognize pathogens is CLRs. CLRs are abundantly expressed on DCs and can bind to the carbohydrate moiety of glycoproteins. CLRs serve as antigen receptors to anchor and internalize antigens and regulate the interaction of DCs with lymphatics and leukocytes. (Geijtenbeek, Kwon, et al., 2000; Geijtenbeek, Torensma, et al., 2000) Moreover, it has been shown that they are involved in the regulation of DC migration. Similar to TLRs, there are different subsets of CLRs expressed by different subsets of DCs. For instance, dendritic cell-specific intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN), also known as CD209, expressed on intDCs, carries multiple functions and interacts with multiple factors, including (i) intercellular adhesion molecule(ICAM)-2, which is expressed on endothelial cells and regulates DC migration to peripheral tissues or LNs (Geijtenbeek, Krooshoop, et al., 2000); (ii) ICAM-3, which is expressed on T-cells and is involved in their priming (Geijtenbeek, Torensma, et al., 2000); and (iii) CD11b/CD18 and carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1), also known as CD66a, that are expressed by neutrophils and contribute to DC activation by neutrophils. (Van Gisbergen et al., 2005)

#### 2.1.3.2.3 NOD-like Receptors

In contrast to TLRs, which are expressed on the plasma membrane or endocytic vesicles of the cells and primarily detect the extracellular microbial products, NLRs are a specialized group of intracellular proteins that recognize intracellular microbial compartments or cellular damage. (K. Murphy & Weaver, 2016) The NLR family includes 23 members in humans and 34 NLR genes in mice. (Franchi et al., 2009) Some NLRs activate NFκB and induce the same inflammatory response as TLR, while other NLRs trigger signalling pathways that induce cell death and yield pro-inflammatory cytokines. (Martinon & Tschopp, 2005)

#### 2.1.3.2.4 Activation of DCs by other immune cells

In addition to PRRs, DC maturation can be simulated either by direct contact with other immune cells, such as neutrophils, or by the products secreted by other immune cells, such as mast cells. For instance, activated mast cells produce granulocyte-macrophage colony-stimulating factor (GM-CSF), TNF-α and IL-4 to activate DCs, neutrophils and macrophages. (Krystel-Whittemore et al., 2016; Malaviya et al., 1996) Among all the cytokines, TNF-α plays a pivotal role in the activation of DCs and T-cells. In vitro treatment of DCs with TNF-α upregulates the expression of CD80, CD86, MHC II and MHC I molecules (Ritter et al., 2003), and *in vivo*, DC maturation and subsequently T-cell activation are diminished in TNF-α-deficient mice. (Trevejo et al., 2001) In addition to mast cells, activated neutrophils and macrophages also secrete a large amount of pro-inflammatory cytokines, such as IL-1, IL-6 and TNF-α (Beauvillain et al., 2007; Iking-Konert et al., 2005; Megiovanni et al., 2006; Van Gisbergen et al., 2005), which, in turn, stimulate DC maturation. Recently it has been shown that neutrophils and DCs also physically interact and scan each other's surface at the site of inflammation, facilitating DC activation upon an infection. (Megiovanni et al., 2006; Van Gisbergen et al., 2005)

### 2.1.3.3 DCs and Adaptive Immunity

DCs are the most abundant antigen-presenting cells in the skin, capturing and transferring antigens to dLNs in order to present them to naïve T-cells. DCs at the steady-state express low to intermediate levels of MHC II, CCR7 and co-stimulatory molecules and have low migratory capacities. However, upon activation, they rapidly upregulate expression of MHC II, co-stimulatory molecules and CCR7 and gain migratory properties to migrate into the nearest dLN. (Banchereau & Steinman, 1998; Malissen et al., 2014) DCs provide three different signals required for T-cells activation: 1) stimulation through TCR; 2) stimulation through co-stimulatory molecules; and 3) secretion of different cytokines. (Banchereau & Steinman, 1998) Interestingly, these signals are different for each DC subset, resulting in a diverse and specific immune response initiated by each DC subset (Figure 4). In addition, based on the type of antigen and tissue, the immune response can vary from a Th1 to Th2 response or tolerance. (Banchereau & Steinman, 1998; Malissen et al., 2014)

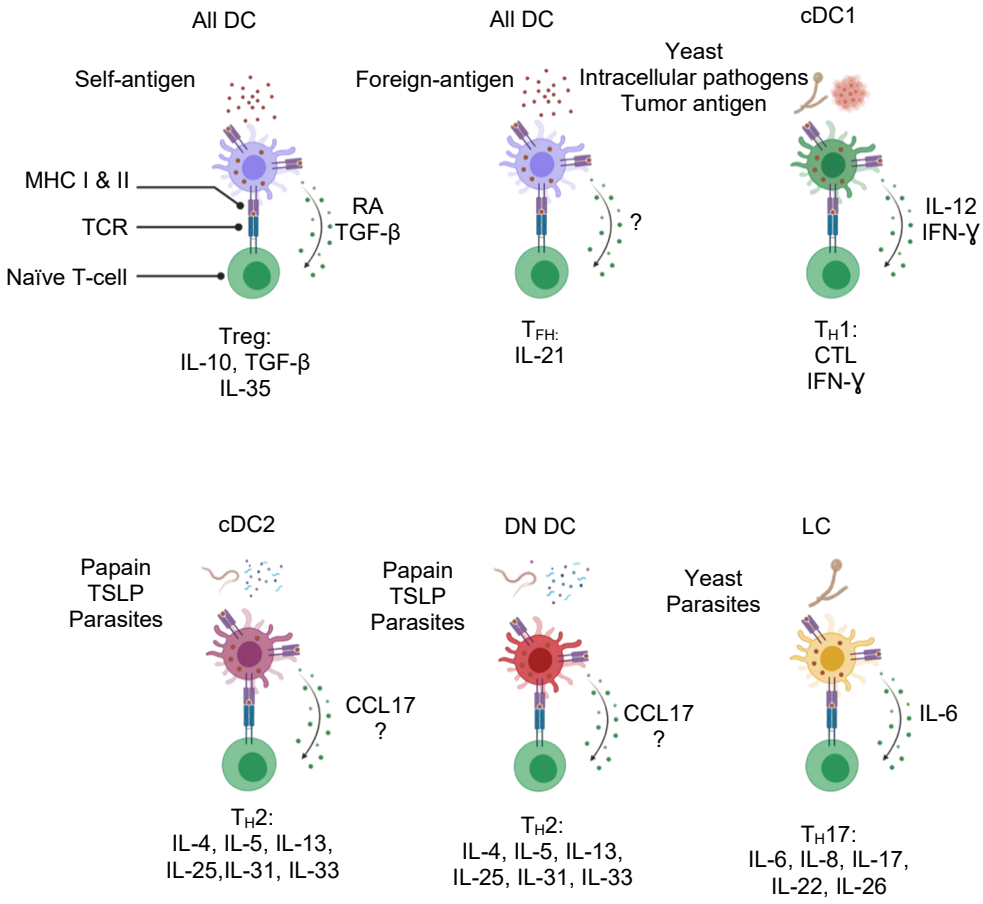


**Figure 4. Schematic presentation of different subsets of dendritic cells (DCs) and their interaction with T-cells.** DCs can induce common and distinct immune responses against an antigen. This scheme illustrates the role of each DC subset in the activation and differentiation of $CD4^+$ and $CD8^+$ T-cells into different effector and memory T-cells. The type of T-cell response is dictated by the type of antigen, tissue, inflammatory cues and type of DC, and the subsequent signals from the DC and local microenvironment to the T-cells. Abbreviations: conventional DC (cDC), major histocompatibility complex (MHC), T-cell receptor (TCR), regulatory T-cell (Treg), interleukin (IL), transforming growth factor beta (TGF-β), retinoic acid (RA), follicular helper T-cell ($T_{FH}$), interferon gamma (IFN-ɣ), cytotoxic T-lymphocyte (CTL), thymic stromal-derived lymphopoietin (TSLP), chemokine (C-C motif) (CCL), T-helper cells ($T_H$), Double-negative DC (DN-DC), Langerhans cell (LC). Figure Modified from the following references: (Akdis et al., 2011; Kashem et al., 2017) This figure was created with www.biorender.com.

### 2.1.3.3.1 Antigen Presentation by DCs

While all nucleated cells can present intracellular antigens through MHC class I molecules to $CD8^+$ T cells, only specific APCs can present extracellular antigens to

$CD8^+$ T-cells to activate them. DCs are one of the most important leukocyte subsets that can capture antigens, process and efficiently present them to T-cells. The process through which the antigen is processed and presented to cytotoxic $CD8^+$ T-cells is referred to as antigen cross-presentation. This process is pivotal for an effective immune response against many pathogens, such as viruses, bacteria, parasites and tumour cells. (Joffre et al., 2012) Two separate pathways have been reported for cross-presentation: (i) the cytosolic pathway, in which the exogenous antigen is degraded and prepared in the cytosol for presentation on MHC I molecules and (ii) the vacuolar pathway, in which antigen preparation takes place within endocytic compartments. (Joffre et al., 2012)

Even though all subsets of DCs, with the exception of LCs (Igyártó et al., 2011), can cross-present an antigen to $CD8^+$ T-cells, cDC1s are superior in cross-presentation of soluble and necrotic cell-derived antigens, especially in the context of TLR3 co-stimulation. (Cruz et al., 2017; Haniffa et al., 2012; Jongbloed et al., 2010) In addition, there is a clear distinction between migratory and resident DCs in T-cell activation in the LNs. Recently, a fascinating intravital imaging study showed that upon local viral infection newly arrived migratory DCs activate $CD4^+$ T-cells but not $CD8^+$ T-cells. In fact, $XCR1^+$ cDC1s only later acquire the antigen and cluster with $CD8^+$ T-cells to activate them. Interestingly, the early activated $CD4^+$ T-cells actively interact with $XCR1^+$ DC1s during their engagement with $CD8^+$ T-cells and possibly licence their activation. (Hor et al., 2015) Conversely, more recently, another group showed that newly arriving migratory $XCR1^+$ cDC1 ($CD103^+$) can interact with $CD8^+$ T-cells more predominantly than the resident $XCR1^+$ DCs ($CD8^+\alpha$) and the migratory DCs that had arrived before immunization. (Kitano et al., 2016) One can envisage that the contrasting results may be due to the type of antigen, timing and location of infection.

#### 2.1.3.3.2 DCs and Th1 Response

DCs are one of the most important regulators of the Th1 response against intracellular pathogens, such as viruses and bacteria. Th1 cells require polarization signals provided by cytokines, such as IL-12 and IL-27. IL-12 is mainly secreted by cDC1s, resulting in the differentiation of naïve $CD4^+$ T-cells into Th1 cells. $Batf3^{-/-}$ mice lacking $CD8\alpha^+$ cDC1s have reduced levels of IL-12 and IFN-ɣ and cannot mount an effective Th1 response against *Toxoplasma gondii*. (Mashayekhi et al., 2011) Similarly, another study showed that $CD8\alpha^-$ cDCs in $Batf3^{-/-}$ mice present the *Leishmania major* antigen to T-cells, instead of $CD8\alpha^+$cDCs, and skew the differentiation of T-cells to a nonprotective Treg and Th2 response, resulting in unresolved and exacerbated pathology with a dramatic increase in parasite load. The same study also showed that $CD103^+$ DCs are the primary source of IL-12 during

*Leishmania major* infection and adoptive transfer of WT cells, but not IL-12p40$^{-/-}$ cells, can recover the anti-*Leishmania major* response. (Martínez-López et al., 2015)

In addition to cDC1s, other subsets of DCs have been shown to initiate Th1 polarization in specific tissues or certain conditions. For instance, it has been shown that submucosal CD11b$^{+}$ DCs can present Herpes simplex virus (HSV) antigens to CD4$^{+}$ T-cells and initiate the Th1 response in vaginal infection with HSV-2. In the same study, the authors showed that CD8α$^{+}$ DCs and LCs were not able to initiate Th1 polarization. (Zhao et al., 2003) cDC2s also present antigen to CD4$^{+}$ T-cells and initiate the Th1 response. During infection with *Mycobacterium bovis* Bacille Calmette-Guérin (BCG) strain, migratory cDC2s (EpCAM$^{low}$ CD11b$^{high}$) transfer the antigen to the dLNs in an IL-1R-I, MyD88, TNFR-I and IL-12p40 dependent manner and present it to CD4$^{+}$ T-cells. (Bollampalli et al., 2015) In addition to cDCs, moDCs are also an important source of IL-12 and can initiate Th1 polarization during infection with *Listeria monocytogenes*. (Zhan et al., 2010)

#### 2.1.3.3.3 DCs and Th2 Response

Th2 cells are important in fighting against parasites and helminths, but can also be very damaging under certain conditions, such as atopic and allergic diseases. Despite the recent progress in identifying the role of DCs in the Th2 response, Th2-polarizing DC subsets and their mechanisms of activation are still largely unknown. Th2 polarization depends on specific cytokines, including IL4, IL-10, IL-25, IL-33 and CCL17. (Fort et al., 2001; Hammad et al., 2003; Jankovic et al., 2004; A. S. MacDonald & Pearce, 2002; Rank et al., 2009) It has recently been shown that upon immunization with fluorescein isothiocyanate (FITC) in dibutyl phthalate, a subset of CD11b$^{+}$ migratory DCs in the dLNs secrete CCL17 to induce a Th2 response. (Kitajima & Ziegler, 2013) Further investigations of the role of DCs in Th2 immunity and their mechanisms of activation showed that thymic stromal lymphopoietin (TSLP), expressed by many cells in the inflamed tissues, and its receptor, TSLP-receptor, cause cDCs to upregulate STAT5 and CCR7 and to secrete CCL17 to initiate Th2 immunity in an animal model of contact hypersensitivity (CHS). (Bell et al., 2013) Similarly, another study showed that CD301b$^{+}$ cDC2s are crucial for the Th2 response during subcutaneous immunization with ovalbumin (OVA), as well as papain or alum. Depletion of these CD301b$^{+}$ cDC2s decreases expression of IL-4 by CD4$^{+}$ T-cells, resulting in a reduction in the number of Th2 cells upon infection with the parasite *Nippostrongylus brasiliensis*. (Kumamoto et al., 2013) Moreover, the transcription factor interferon regulatory factor 4 (IRF4) mediates the differentiation of cDC2s and its Th2-inducing functions during infection with *Nippostrongylus brasiliensis* and immunization with a protease allergen. (Gao et al., 2013) Together, these results indicate an important role of

CD301b$^+$ DC2s in Th2 immunity. Other subsets of DCs can also have a regulatory effect on Th2 immune responses. For instance, a subset of CD103$^+$ DCs constitutively expresses high levels of IL-12, by which they actively suppress Th2 immune responses .(Everts et al., 2016)

#### 2.1.3.3.4 DCs in Th17 Response

Th17 response is essential in host defence against many extracellular antigens, such as fungal and bacterial pathogens. Different cytokines, including IL-6, TGF-β, IL-23 and IL-1β, can differentiate and support the maturation of Th17 cells. (Korn et al., 2009) LCs play a critical role in this type of response. Upon *Candida albicans* skin infection in mice, LCs, but not Langerin$^+$ dDCs, present *Candida albicans* antigen and initiate the Th17 immune response by producing a large quantity of Th17-differentitating cytokines, including TGF-β, IL-1β and IL-6. In contrast, Langerin$^+$ dDCs initiate Th1 cell polarization, prime CTLs and inhibit Th17 cell responses. (Igyártó et al., 2011)

Recently, MyD88 signalling has been shown to be crucial for antigen-specific Th17 responses. Langerin-Cre MyD88$^{fl}$ mice infected with *Candida albicans* have impaired Th17 cells and lower levels of IL-1β, IL-6 and IL-21, suggesting a critical role of MyD88 signalling in full activation of LCs during fungal infections. (Haley et al., 2012) LCs are also involved in generating a subset of CD4$^+$ T-cells that secrete IL-17 upon infection with *Staphylococcus aureus*. (T. Kobayashi et al., 2015)

In addition to LCs, IL-6 secreted by CD301b$^+$ cDC2s as well as TGF-β1 secreted by other subsets of DCs have been shown to be important in generating Th17 cells during infection with *Streptococcus pyogenes.* (Linehan et al., 2015) Interestingly, even though other subsets of DCs, such as CD103$^+$ DCs, do not directly initiate the Th17 response, they can prime a subset of CD8$^+$ T-cells (Tc17) that secrete IL-17 and initiate the Th17 response during infection with *Staphylococcus epidermidis* in mice. (Naik et al., 2015)

#### 2.1.3.3.5 DCs and $T_{FH}$ response

$T_{FH}$ cells are a subset of CD4$^+$ T-cells that collaborate with B-cells to initiate humoral responses against pathogens. (Krishnaswamy et al., 2018) Like other T-cell responses, DCs also play a pivotal role in generating $T_{FH}$ cells. LCs have been shown to play an important role in $T_{FH}$ differentiation and humoral response (Kumamoto et al., 2016) and are involved in IgG1 production during patch-immunized ovalbumin. (Ouchi et al., 2011) Depletion of LCs decreases IgE production at the steady-state and in response to protein antigens. (Nakajima et al., 2012) Interestingly, selective depletion of LCs decreases the size of GCs and significantly reduces the $T_{FH}$

response and the antibody response to *Leishmania major* infection. (Zimara et al., 2014)

Induction of the $T_{FH}$ and humoral responses is not restricted to only LCs as other DC subsets are also involved. It has been shown that the depletion of either LCs or both LCs and cDC1 does not completely abrogate the $T_{FH}$ and GC B-cell response in mice infected with HIV p24-coated nanoparticles, suggesting that both subsets, as well as other subsets of DCs, contribute to the humoral response. In fact, moDCs are even more efficient in supporting GC formation than cDCs. (Levin et al., 2017) Corroborating these results, an earlier study showed that $CD103^+$ cDC1s are sufficient to initiate $T_{FH}$ cell development and antibody production in mice at the steady-state. However, these $CD103^+$DCs are less efficient in priming $T_{FH}$ and humoral responses than LCs. (Yao et al., 2015)

On the other hand, not all DCs can promote $T_{FH}$ responses. Indeed, some of the DCs subsets may have a regulatory effect on the $T_{FH}$ response and antibody production. For instance, selective depletion of $CD301^+$ DCs significantly enhances the $T_{FH}$ and GC B-cell responses leading to a robust antibody production against protein antigens. (Kumamoto et al., 2016)

#### 2.1.3.3.6 DCs in Tolerance

Even though activation of the immune response is necessary for eliminating invading pathogens, its activation against the self-antigens can be very harmful. Therefore, immune cells with strong self-reactive receptors must be eliminated to prevent autoimmune responses. Negative selection of autoreactive immune cells in the bone marrow and thymus is one of the ways to induce self-tolerance. In the thymus, DCs negatively select and eliminate autoreactive T-cells. The autoreactive cells that escape the central tolerance must be eliminated later in the secondary lymphoid organs or peripheral tissues. In these tissues, DCs also play an important role in inducing tolerance and removing autoreactive T-cells from the repertoire. (K. Murphy & Weaver, 2016)

It was initially thought that at the steady-state and in the absence of antigen, DCs induce tolerance as their alternative default role in immunity. However, recently it has been shown that tolerance induction by DCs is an active process in which semi-mature DCs with medium expression levels of MHC II and CCR7 gain migratory properties to migrate into the dLNs and induce tolerance. This process is selectively mediated by various mechanisms, such as alteration in E-cadherin-mediated DC-DC adhesion. (Jiang et al., 2007; Manicassamy et al., 2010) NF-κB signalling is also crucial for the migration of DCs into the dLNs and tolerance induction at the steady-state. (Baratin et al., 2015) DCs also secrete several molecules and cytokines, such

as retinoic acid (RA) (Hill et al., 2008) and TGF-β (Marie et al., 2005; L. Zhou et al., 2008), to polarize T-cells towards Tregs.

## 2.2 Secondary lymphoid tissues – a bridge between innate and adaptive immunity

One of the most compelling narratives in immunology is the activation of the adaptive immune response in the secondary lymphoid organs. The immune cells in peripheral tissues constantly survey their environment for pathogens and upon encountering a pathogen, the innate immune response is initiated to eliminate the pathogen. If this is not successful, the adaptive immune response is subsequently initiated to overcome it. One of the most crucial components of the adaptive immune system is the lymphatic system. The lymphatic system consists of primary and secondary lymphoid organs as well as lymphatic vessels. The primary lymphoid tissue consists of the thymus and bone marrow, while the secondary lymphoid organs include the LNs and the spleen. The lymphatic vessels connect the peripheral tissues to the LNs and the LNs to each other and eventually to the Thoracic duct.

In addition to the lymphatic vessel. peripheral tissues contain a network of blood vessels which distribute oxygen, nutrients, and immune cells throughout the periphery. Blood vessels can be classified into arteries, capillaries, or veins. While the arteries carry away blood from the hurt, capillaries distribute the blood within the peripheral tissues, and veins eventually carry the blood back to the heart. (Tucker et al., 2021) Under normal conditions, the flow of nutrients and migration of leukocytes into the peripheral tissues is limited. However, upon inflammation, the permeability of the capillaries is increased allowing more nutrients and oxygens to be delivered to the inflamed tissues. (Wilhelm & Mason, 1960) Moreover, different adhesion molecules are upregulated on the endothelium of blood vessels in the inflamed tissues to regulate the migration of leukocytes into the inflamed tissues. (Nourshargh & Alon, 2014)

### 2.2.1 Lymphatic vessels and molecules involved in DC migration from periphery to lymph nodes

The lymphatic vessels are one of the most important compartments of the lymphatic system. Lymphatic vessels consist of afferent and efferent lymphatic vessels. The central role of the afferent lymphatic vessels is to collect interstitial fluid, macromolecules and immune cells from the peripheral tissue and transfer them to the dLNs. In contrast, efferent lymphatic vessels exit the LN and eventually fuse with other efferent lymphatic vessels to form the thoracic duct and right lymphatic duct. The prime role of efferent lymphatic vessels is to connect the lymphatic system

to the cardiovascular system to return the interstitial fluid and immune cells back to the bloodstream. (Ulvmar & Mäkinen, 2016)

Lymphatic vessels can be characterized by the expression of the vascular endothelial growth factor receptor-3 (VEGFR-3) (Kaipainen et al., 1995), the lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1) (Banerji et al., 1999), the mucin type-1 protein podoplanin (Breiteneder-Geleff et al., 1999), and Prospero homeobox protein 1 (PROX-1). (Wigle & Oliver, 1999) Peripheral lymphatic vessels are blind-ended and are known as capillary lymphatic vessels. Capillary lymphatic vessels are then merged into bigger vessels and form collecting lymphatic vessels. The collecting lymphatic vessels eventually fuse and form the afferent lymphatic vessels, which are connected to the LN (Figure 5). (Lauweryns & Boussauw, 1973; Schulte-Merker et al., 2011)

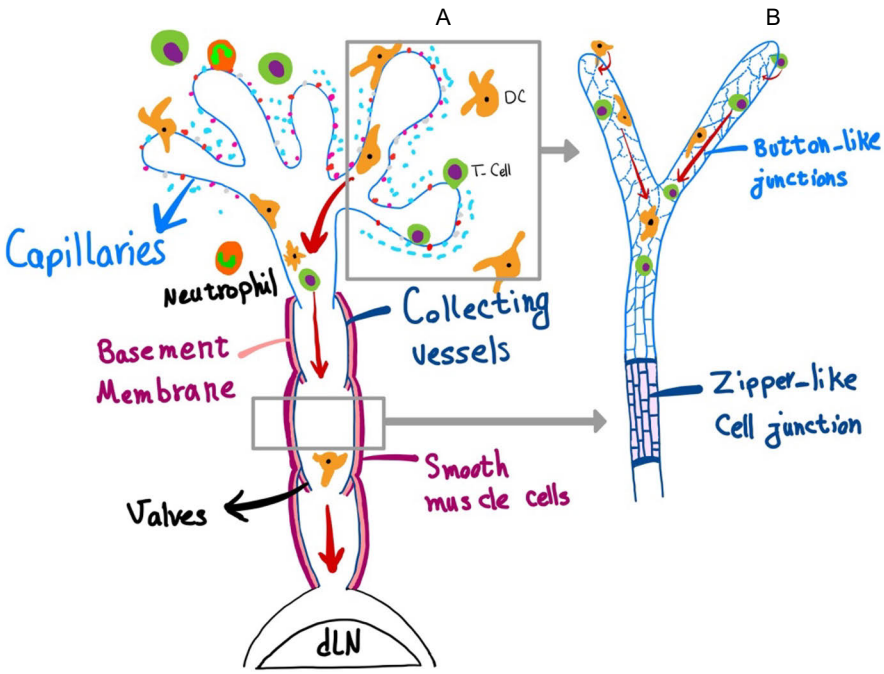


**Figure 5. Structure of capillary and collecting lymphatic vessels.** The capillary lymphatic vessels can be distinguished by the button-like junctions, lack of smooth muscle and discontinuous basement membrane, high expression of lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1) and low expression of podoplanin. Leukocytes migrate into the lymphatic vessels through the button like-junctions. The structure of capillary lymphatic vessels allows them to absorb fluid and soluble molecules from the interstitial space. In contrast to capillaries, collecting lymphatic vessels can be recognized by zipper-like junctions, smooth muscle coverage, continuous basement membrane and intraluminal valves. Collecting lymphatic vessels also express low levels of LYVE-1, but high levels of podoplanin. Abbreviation: Dendritic cell (DC), draining lymph node (dLN). Figure Modified from the following references: (Hunter et al., 2016; Teijeira et al., 2014)

Capillary lymphatic vessels are surrounded by a discontinuous basement membrane and lack smooth muscles. By contrast, collecting lymphatic vessels are covered by smooth muscle cells and basement membrane. (Schulte-Merker et al., 2011; Ulvmar & Mäkinen, 2016) Collecting lymphatic vessels are also equipped with intraluminal valves to prevent bidirectional lymph flow (Figure 5). (Lauweryns & Boussauw, 1973) Another critical distinction between capillaries and collecting lymphatic vessels is their cell junctions. Capillary LECs are oakleaf-shaped and are joined by discontinuous button-like junctions, which form a loose flap of about 2-3µm, called "flap valves", also known as primary valves, through which leukocytes can enter into the lymphatic vessels. These flap valves together with the discontinuous basement membrane and lack of smooth muscles make the capillary vessels permeable and enable them to absorb fluid and soluble molecules from peripheral tissues. (Baluk et al., 2007) Recently, with the help of advanced imaging techniques, it has been shown that the flap valves are transiently dilated by the transmigrating DCs. (Pflicke & Sixt, 2009) In contrast, collecting lymphatic endothelial cells are connected by continuous zipper-like junctions, which together with continuous basement membrane and perivascular smooth muscles, make these vessels relatively impermeable (Figure 5). (Baluk et al., 2007)

In contrast to leukocyte migration from the blood vessels, which involves extravasation, leukocyte migration into afferent lymphatic vessels involves intravasation. Due to the loose cell-cell contacts and lack of a continuous basement membrane in capillary lymphatic vessels, it was initially thought that leukocytes passively enter the lymphatics with interstitial fluid flow. However, recently it has been shown that leukocyte migration into the lymphatics is an active process, which occurs sequentially, and many adhesion molecules and chemokines are involved in this process (Table 2). (Nourshargh et al., 2010; Vicente-Manzanares et al., 2009) In the first step, leukocytes migrate a few hundred micrometres through the surrounding interstitial tissues to access the initial capillary lymphatic vessels. Leukocytes then need to traverse through the basement membrane before engaging with the surface of initial capillary lymphatic vessels and transmigrate through the endothelium to enter the vessel lumen. (S. Wang et al., 2006) After intravasation, leukocytes crawl on the lumen side of the capillary lymphatic vessels to enter the collecting lymphatic vessels followed by cellular detachment, and are then passively carried with the lymph flow to the dLNs. (Jackson, 2019) Interestingly, leukocytes have been shown to actively interact with lymphatics both before transmigrating into the lumen as well as when probing the lumen while migrating to the dLNs. (Nitschké et al., 2012; Sen et al., 2010; Tal et al., 2011)

**Table 2. Molecules involved in DC migration from the peripheral tissues.**

| Molecule | Ligand/Mechanism | Reference |
|---|---|---|
| **CCL19/CCL21** | CCR7 | (Förster et al., 1999; Russo et al., 2016; Saeki et al., 1999) |
| **ICAM-1 & VCAM-1** | Integrins MAC-1 and LFA-1 | (Camponeschi et al., 2019; Johnson et al., 2006; Lämmermann et al., 2008) |
| **CX3CL1** | CX3CR1 | (Johnson & Jackson, 2013) |
| **CXCL12** | CXCR4 | (Kabashima et al., 2007) |
| **CCL1** | CCR8 | (Jakubzick et al., 2006; Qu et al., 2004) |
| **JAM-A/JAM-C** | Integrins αMβ2 Mac-1 and VLA-4 | (Ballet et al., 2014; Cera et al., 2004; Santoso et al., 2002) |
| **Podoplanin/CLEC-2** | CLEC-2 | (Acton et al., 2012; Bianchi et al., 2017) |
| **PD-L1** | Signalling | (Lucas et al., 2020) |
| **LYVE-1** | Hyaluronan (HA) | (Johnson et al., 2017, 2021) |
| **Semaphorin3a (Sema3a)** | Actomyosin contraction via Plexin-A1 & Neuropilin | (Takamatsu et al., 2010) |
| **S1P** | S1P1/S1P3 | (Czeloth et al., 2005; Jackson, 2019; Rathinasamy et al., 2010) |
| **Prostaglandin receptors** | Control MMP-9 expression /CCR7 signalling | (Scandella et al., 2004; Yen et al., 2008) |
| **Metalloproteases (MMP)** | Via PGE2 | (Ratzinger et al., 2002; Yen et al., 2008) |
| **ALCAM** | Galectin-8, L1CAM | (Iolyeva, Karaman, et al., 2013; Maddaluno et al., 2009; Willrodt et al., 2019) |
| **Rho-associated protein kinase (ROCK)** | Impairs intralymphatic DCs crawling | (Nitschké et al., 2012) |
| **ACKR4** | Scavenging CCL19/21 | (Bryce et al., 2016) |
| **Clever-1** | Unknown | (Tadayon et al., 2021) |
| **PECAM-1 (CD31) and CD99** | Unknown | (Torzicky et al., 2012) |

At steady-state, the number of migratory leukocytes at sites, such as skin, is very low. (Jackson, 2019) The main leukocyte population present in afferent lymphatic vessels consists of T-cells, DCs, macrophages, neutrophils, and other rare immune cells, such as B-cells, monocytes and mast cells. (Kabashima et al., 2019; Sokolowski et al., 1978; Tong et al., 2015; Yawalkar et al., 2000) Among the leukocytes, T-cells are the most abundant leukocytes (80-90%) in the lymph. These

T-cells mostly consist of antigen-experienced $CD4^+$ $CD45RO^+$ effector memory T-cells ($T_{EMs}$), $FOXP3^+$ $T_{regs}$, and to a lower extent, $CD8^+$ T-cells. Naïve T-cells rarely exist in afferent lymphatic vessels, mainly because they rarely migrate into peripheral tissues and mostly migrate between the secondary lymphoid organs and blood circulation. (Bromley et al., 2005, 2013; Hunter et al., 2016) Even though DCs are the second most abundant leukocytes in the afferent lymphatics, their number is still low during homeostasis. (Huang et al., 2000; Ohl et al., 2004; Scheinecker et al., 2002) Many chemokines and adhesion molecules are involved in leukocyte intravasation and their journey to the LNs.

#### 2.2.1.1 CCL19/CCL21

CCL19/CCL21 and their receptor, CCR7, are the most important regulators of leukocyte migration into afferent lymphatic vessels and the LNs. CCL19 and CCL21 are expressed on afferent lymphatic vessels, LECs and HEVs, and their receptor CCR7 is expressed on T-cells, DCs, monocytes and neutrophils. (Förster et al., 1999; Russo et al., 2016; Sallusto et al., 1998) There are two types of CCL21: (i) CCL21-Leu and (ii) CCL21-Ser.

CCL21-Leu is mainly expressed on collective lymphatic endothelium, whereas CCL21-Ser is expressed on a subset of lymphatic endothelium, HEV and by stromal cells in T-cell zones of the LNs. (Nakano & Gunn, 2001; Vassileva et al., 1999) CCL21-Ser is involved in guiding T-cells and DCs towards the T-cell zone in the LNs, while CCL21-Leu is responsible for attracting DCs to afferent lymphatics in peripheral tissues. (S.-C. Chen et al., 2002; Förster et al., 2012; Vassileva et al., 1999) Interestingly, DCs in mice lacking CCL21-Ser, but normal in CCL21Leu expression, can still enter the dermal afferent lymphatics and migrate to the LN, but are unable to enter the LN and locate themselves in the T-cell zone, indicating a crucial role for CCL21-Ser in guiding DCs and T-cells to the T cell zone. (Luther et al., 2000)

After the binding of DCs to surface-bound CCL21, DCs proteolytically cleave the heparan sulfate-binding moiety and generate a soluble form of CCL21. (Schumann et al., 2010) This soluble CCL21 polarises and attracts leukocytes toward lymphatic vessels. Recently, an elegant imaging study showed the importance of the CCL21 gradient in guiding DCs towards the lymphatics. Using an intravital imaging approach, Weber and colleagues demonstrated that interstitial DC migration is random and in all the directions in peripheral tissues until they reach the distance of approximately 90 µm from the initial capillary lymphatic vessels. Thereafter, the CCL21 gradient directionally guides them towards lymphatic vessels. (Weber et al., 2013)

The surface-bound CCL21 mediates DC migration by activating integrins and adhesion molecules. (P. M. Murphy, 2010) Recently, it has been shown that CCL21 also plays a role in intralymphatic DC crawling. Russo et al. showed that blocking of CCL21 abolishes intralymphatic DC crawling from the capillary towards the collecting lymphatic vessels and subsequently reduces DC migration to the dLNs. (Russo et al., 2016)

In addition to DCs, other types of immune cells also use CCL19/21 to migrate. Lymphocytes use the CCR7-CCL19/21 axis to enter afferent lymphatic vessels. (Bromley et al., 2005; Debes et al., 2005; Förster et al., 1999) During immune surveillance and acute inflammation, CCR7 regulates the transit of T-cells into afferent lymphatic vessels, and T-cells that lack CCR7 fail to migrate into the dLNs. (Bromley et al., 2005; Debes et al., 2005) However, the role of CCR7 and CCL21 in T-cell exit from the periphery might be context-dependent, as it has been shown that the efficient egress from chronically inflamed skin is CCR7-independent. (M. N. Brown et al., 2010) Neutrophils also express CCR7 and use it to enter the afferent lymphatic vessels. However, it has remained controversial whether neutrophil migration via the lymphatics is CCR7-dependent. While Hampton and colleagues show that the neutrophil egress from the skin is CCR7-independent (Hampton et al., 2015), others have shown that neutrophil entry into lymphatic vessels is strictly CCR7-dependent. (Arokiasamy et al., 2017; Beauvillain et al., 2011) Monocytes also use CCR7 to enter afferent lymphatic vessels and migrate to the LNs. (Becker et al., 2013; Jakubzick et al., 2013)

#### 2.2.1.2 ICAM-1

ICAM-1, also known as CD54, is a cell-surface glycoprotein, which is constitutively expressed in blood and lymphatic endothelial cells. ICAM-1 regulates the migration of leukocytes from peripheral tissues into dLNs through afferent lymphatic vessels. (Issekutz & Issekutz, 1992; Johnson et al., 2006; Teijeira et al., 2017) ICAM-1 binds to αM-β2 integrin, also known as Macrophage-1 antigen (MAC-1) and CD11b/CD18, and lymphocyte function-associated antigen 1 integrin (LFA-1, CD11a/CD18), expressed on the surface of many leukocytes. (Issekutz & Issekutz, 1992; Johnson et al., 2006) Due to this binding, it was initially thought that MAC-1 and LFA-1 function as ligands and regulate DC migration. However, ablation of integrin heterodimers from DCs surprisingly showed that integrins do not contribute to the transmigration of DCs or any other leukocytes at steady-state. (Lämmermann et al., 2008) This apparent discrepancy may be due to very low expression levels of ICAM-1 at steady-state. In support of this explanation, it has been recently shown that ICAM-1 enriched microvilli structures are formed around DCs that adhere to inflamed skin lymphatic vessels in a β-integrin-dependent manner and accompany

the migrating DCs throughout their transmigration. Moreover, these microvilli structures are rarely formed in normal conditions, indicating a role of integrins only under inflammatory conditions. (Teijeira et al., 2013) Interestingly, these LEC microvilli structures are formed in the vicinity of CCL21 depots and blocking of CCL21 interrupts the formation of these microvilli structures. (Teijeira et al., 2013) In addition to DCs, T-cells use LFA-1 and ICAM-1 to migrate into the LNs. (Teijeira et al., 2017) ICAM-1 also regulates the velocity of intralymphatic crawling of T-cells. Moreover, blocking of ICAM-1 or its ligand, LFA-1, decreases the velocity of crawling T-cells within the afferent lymphatic vessels. (Teijeira et al., 2017)

Another subset of leukocytes that uses ICAM-1 and its ligands to exit from peripheral tissues and migrate to dLN is neutrophils. Neutrophils are considered short-lived differentiated phagocytes with a life span of 1 to 5 days in blood circulation (Coxon et al., 1999; Gorlino et al., 2014; Hampton et al., 2015; S. D. Kobayashi, 2005; Rigby et al., 2015). Thus, it initially was believed that following neutrophil migration into peripheral tissues, they undergo apoptosis and are engulfed by the other phagocytes. (Cox et al., 1995; Haslett, 1992) However, it has been shown that a subset of the neutrophils that migrated to the inflamed tissue as the first line of defence can migrate to the dLNs via afferent lymphatic vessels and carry antigens. (Gorlino et al., 2014) Recently, Arokiasamy et al. showed that blocking of ICAM-1 or its neutrophil-expressed ligand, MAC-1, inhibits neutrophil crawling along the lumen of afferent lymphatic endothelium. Interestingly, they also showed that the intraluminal neutrophil crawling within the afferent lymphatic endothelium is regulated by TNF-α. (Arokiasamy et al., 2017) More interestingly, TNF-α upregulates ICAM-1 upon inflammation. In addition to TNF-α, IL-1 and IFN-ɣ also upregulate the expression of ICAM-1 via the NF-κB pathway. (Roebuck & Finnegan, 1999)

#### 2.2.1.3 CXCR4/CXCL12 and CX3CR1/CX3CL1

CXCR4 expressed on activated cutaneous DCs, and its ligand, CXCL12 expressed on afferent lymphatic vessels, mediate DC migration to the lymphatic vessels. (Kabashima et al., 2007) Blockade of CXCR4 expressed by DCs significantly reduces the migration of DCs into the dLNs. Blockade of CXCR4 also significantly reduces the CHS response in mice, indicating an important role for CXCR4 in DC migration and initiation of the adaptive immune response. (Kabashima et al., 2007) In addition to DCs, CXCR4 is also expressed on a subset of T-cells and is involved in T-cell trafficking into different organs, including the bone marrow. (Goedhart et al., 2019) However, blockade of CXCR4 does not affect T-cell egress from inflamed skin, suggesting that this receptor might not be involved in T-cell egress from peripheral tissue. (Geherin et al., 2014) Since CXCR4 is expressed on neutrophils, it

was thought that CXCR4 regulates neutrophil egress from peripheral tissues, in a similar manner than in T cells. However, Arokiasamy et al. showed that CXCR4 blockade does not significantly affect neutrophil egress into lymphatic vessels upon inflammation. (Arokiasamy et al., 2017)

Another important transmembrane chemokine involved in DCs transit into the LNs via lymphatics is C-X3-C motif chemokine ligand 1 (CX3CL1). Lymphatic endothelial cells secrete CX3CL1 in response to inflammatory stimuli, and rather than being a membrane-anchored chemokine, CX3CL1 guides DCs toward lymphatic vessels. (Johnson & Jackson, 2013) CCL21, expressed at lower levels on the collector lymphatics than capillary lymphatics, regulates DC migration into the capillary lymphatic vessels. Recently, it has been shown that CXCL12 and CX3CL1 are upregulated on the collecting LVs upon inflammation and are likely to act in concert with CCL21 in recruiting DCs into the collecting lymphatics. (Arasa, Collado-Diaz, Kritikos, et al., 2021) Considering DC migration into the collectors with low levels of CCL21 expression (Russo et al., 2016), it can be envisaged that CXCL12 and CX3CL1 might have a role in DC transmigration into the collecting LVs in inflammation. (Arasa, Collado-Diaz, & Halin, 2021)

#### 2.2.1.4 Podoplanin

Podoplanin is a small transmembrane glycoprotein expressed on LECs and follicular reticular cells (FRCs) of secondary lymphoid organs. (Breiteneder-Geleff et al., 1999; Farr et al., 1992) Podoplanin is involved in maintaining vascular integrity (Herzog et al., 2013) and the prenatal deletion of podoplanin causes death due to respiratory failure. (Ramirez et al., 2003) Recently it has been shown that postnatal deletion of podoplanin leads to blood-filled lymphatic vessels (Acton et al., 2012) and it impairs DC migration into the dLNs. (Bianchi et al., 2017) In addition, the ligation of C-type lectin-like receptor 2 (CLEC-2) expressed on DCs with podoplanin is crucial in DC migration into the dLNs. (Acton et al., 2012)

#### 2.2.1.5 LYVE-1

LYVE-1 is a type I integral membrane glycoprotein that binds to a large ubiquitous glycosaminoglycan polymer hyaluronan (HA). (Banerji et al., 1999) LYVE-1 is abundantly expressed in lymphatic capillaries and their button-like junctions, whereas it is largely absent from collecting lymphatics and their zipper-like junctions. (Ulvmar & Mäkinen, 2016) Ligation of LYVE-1 and HA on DCs promotes DC transmigration into the capillary lymphatics. Deletion of LYVE-1 or the hyaluronan coat significantly reduces immediate DC migration into the dLNs upon inflammation and decreases their capability to prime $CD8^+$ T-cells in the dLNs.

(Johnson et al., 2017) In addition to DCs, macrophages also express HA and use LYVE-1 to transmigrate across the lymphatic endothelium. (Lawrance et al., 2016)

#### 2.2.1.6 Semaphorins

Semaphorins are a group of transmembrane proteins and secreted proteins that were initially identified as axonal guidance molecules. (Kolodkin et al., 1993) However, until recently, the primary role of semaphorins in DC migration has remained unknown. A recent study showed that lymphatics produce semaphorin 3A, and its ligation with plexin-A1–neuropilin 1 (NRP1) receptor complex, expressed by DCs, is involved in the transmigration of DCs into the LNs. (Takamatsu et al., 2010) Moreover, lymphatic-derived semaphorin 3A induces actomyosin contraction to generate a contractile force as DCs pass through narrow gaps and transmigrate across the lymphatic endothelium. (Takamatsu et al., 2010)

### 2.2.2 Lymph Node (LN)

LNs are encapsulated bean-shaped organs located along the entire lymphatic system and they contain lymphocytes, antigen-presenting cells (APCs) and other immune cells. LNs consist of the cortex, paracortex and medulla, enclosed by a capsule (Figure 6). The cortex area consists of follicles, where B-cells and FDCs reside to interact and initiate humoral immune responses. The follicles are separated from the other regions by the interfollicular cortex. The paracortex area (deep cortex) has a central and peripheral region. In the central region (central deep cortical unit, central DCU), T-cells and DCs are located and interact with each other. The peripheral region (peripheral deep cortical unit, peripheral DCU) and the interfollicular cortex act as a transit corridor, as it contains the high endothelial venules (HEVs), arterioles and paracortical sinuses. The medulla consists of medullary sinuses surrounding medullary cords, where plasma cells home. Immature plasma cells migrate to medullary cords to mature and, upon maturation, secrete antibodies into the lymph that exists the LNs via the efferent lymphatics. (Gretz et al., 1996, 1997; Willard-Mack, 2006) There are two paths through which leukocytes can enter LN: (i) from the bloodstream and by crossing the HEVs and (ii) by migrating from peripheral tissues with lymph via the afferent lymphatic vessels. Lymph, which contains leukocytes and free antigens, enters the LNs through multiple afferent lymphatic vessels and traverses through sinuses, passing through the cortex, paracortex and eventually flowing through medullary sinuses and exiting via the efferent lymphatic vessels to finally return to the bloodstream via the thoracic duct. (Gretz et al., 1996, 1997; Willard-Mack, 2006)

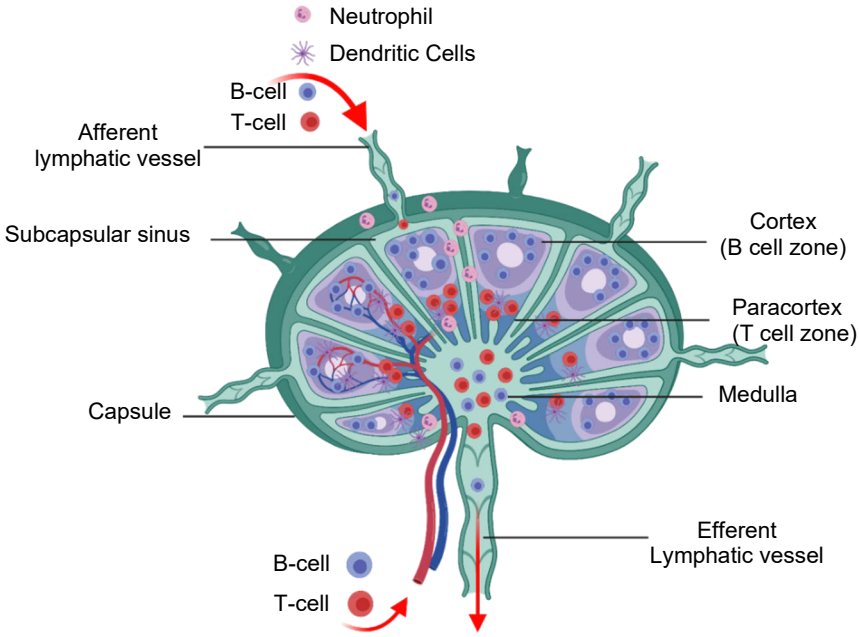


**Figure 6. A schematic representation of the lymph node structure.** The lymph node cross section shows different regions of a lymph node, including afferent and efferent lymphatics, cortex, paracortex, medulla, capsule and subcapsular sinus. Figure Modified from Murphy et al. (K. Murphy & Weaver, 2016) This figure was created with www.biorender.com.

#### 2.2.2.1 Lymph node stromal cells (LNSCs)

Upon inflammation immune cells actively migrate to different regions of LNs and interact with each other to activate adaptive immune responses. This interaction between immune cells occurs against the background of lymph node stromal cells (LNSCs). The LNSCs consists of blood endothelial cells (BECs), fibroblastic reticular cells (FRCs), LECs, and myofibroblastic pericytic cells (pericytes). (Malhotra et al., 2012)

FRCs include multiple subsets falling into four cell types: (i) T-cell zone FRCs (TRCs), (ii) follicular dendritic cells (FDCs), (iii) marginal reticular cells (MRCs), and (iv) medullary FRCs (medRCs) (Figure 7). (Krishnamurty & Turley, 2020) Each subset has a distinct location in the LNs. For instance, TRCs are typically located in the paracortex and define the T-cell zone and intrafollicular regions between the B-cell and T-cell zones. On the other hand, FDCs are commonly located in the cortex and B-cell zone. MRCs are situated in the cortex area, between the subcapsular sinus and the B-cell follicles. (Krishnamurty & Turley, 2020; Perez-Shibayama et al., 2019)

Historically, LECs in LNs are categorized by their location within the LNs. Subcapsular sinus LECs (S-LECs) are localized on the outer side of LNs and form a narrow sinus where they can closely interact with leukocytes and molecules that enter the LN from afferent lymphatics. (Kedl & Tamburini, 2015) Cortical LECs (C-LECs) form the blunt-ended vessels that branch into the T cell zone between B-cell follicles and are situated adjacent to HEVs. (Grigorova et al., 2010; Kedl & Tamburini, 2015; Sinha et al., 2009) Finally, medullary LECs (M-LECs) are located within the medulla of LNs and collect lymphoid fluid from the LNs into efferent lymphatic vessels. (Tewalt, Cohen, Rouhani, & Engelhard, 2012)

#### 2.2.2.1.1 Role of LNSCs in leukocyte homeostasis

LNSCs plays a fundamental role in maintaining homoeostasis of immune cells in the LNs. Each subset of LNSCs express a specific range of adhesion molecules and cytokines and consequently has specific characteristics to mediate leukocyte trafficking to and within the LNs. All the LNSCs, with the exception of pericytes and BECs, are characterized by high expression of podoplanin. Podoplanin maintains the reticular tension, LN stiffness and increases and maintains the integrity of HEVs by regulating actomyosin contractility in FRCs. (Astarita et al., 2015) Mice deficient in podoplanin lose their HEV integrity and its barrier function and consequently exhibit spontaneous and severe bleeding in the mucosal lymph nodes. (Herzog et al., 2013) Each subset of LNSCs has a distinguishing characteristic and play a certain role in leukocyte trafficking into the LNs. In the following chapters, I further discuss different types LNSCs in more detail.

##### FRCs

One of the most important LNSCs involved in homeostasis is FRCs. Initially, it was believed that the FRC network is merely a physical barrier to lymphocyte migration. However, recently many studies have shown that FRCs orchestrate the migration of lymphocytes and DCs into the different compartments of LN by secreting a wide range of chemokines and cytokines, including CXCL2, CXCL9, CXCL10, CXCL12, CXCL13, CCL19, CCL21, IL-7 and IL-33. (Bajénoff et al., 2006; F. D. Brown & Turley, 2015; Nakayama & Bromberg, 2012) Among all these chemokines, CCL19 and CCL21 are the most crucial chemokines involved in leukocyte migration into and within the LNs. TRCs and LECs of subcapsular sinus (SCS) are the primary sources of CCL19 and CCL21 in the LNs. After arriving in the LNs, CCR7-expressing DCs and T-cells follow the gradient of CCL19 and CCL21 towards and into the T-cell zone (Figure 7). (Ulvmar et al., 2014) Interestingly, to enforce DC migration into the T-cell zone after their arrival in the SCS of LNs, ceiling LECs

express the atypical CCL19 and CCL21 chemokine receptor 4 (ACKR4). ACKR4 scavenges chemokines from the sinus lumen to shape the abundance gradient of chemokines across the sinus floor. (Ulvmar et al., 2014) HEVs also express CCL21 and CCL19 to regulate the migration of CCR7-expressing cells into the LN (Figure 7). (Luther et al., 2000, 2002)

### TRCs and LECs

TRCs and LECs also play a pivotal role in homeostasis. TRCs and LECs are the main sources of IL-7 and CCL19 in LNs to attract naïve T-cells and support their survival (Figure 7). (Link et al., 2007) IL-7 is also essential for LN remodelling after viral infection and LN reconstruction after vascular transplantation. (Onder et al., 2012) LECs also express IL-7 receptor α chains (IL-7Rα), which together with their IL-7 secretion can support lymphangiogenesis and lymphatic drainage from the periphery into the LNs. (Iolyeva, Aebischer, et al., 2013)

In addition to the expression of chemokine and adhesion molecules, the hollow structure of the FRC network allows them to form a conduit system to convey soluble antigens and molecules to the different compartments of LNs. (Gretz et al., 2000) Considering the structure of the conduit system and the direction of lymph flow from the afferent LVs towards and into the subcapsular sinus, medullary sinus, and efferent lymphatic vessels, it was initially envisaged that lymph-born material that enters the subcapsular sinus can reach different compartments of the LNs without any barrier. However, Gretz et al. showed that s.c. injected antibodies and dextrans with high molecular weight are visible in the subcapsular and medullary sinuses but cannot reach the cortex and paracortex. Moreover, they showed that molecules with low molecular weight, such as chemokines, have limited access to the parenchyma and cortex via the reticular conduit network, and this occurs in a very specific manner. (Gretz et al., 2000) Surprisingly, contrary to previous studies, recently Kähäri et al. showed that large biomolecules and lymph-born proteins, such as antibodies, are traversed across the subcapsular sinus by fluid-phase transcytosis and can reach the parenchyma of the lymph node in an intact form in seconds. (Kähäri et al., 2019)

### FDCs and MRCs

FDCs predominate the B-cell zone and secrete large quantities of CXCL13 and B-cell survival factor BAFF to guide CXCR5-expressing B-cells into the follicles and support their maturation, survival and proliferation. FDCs also express complement receptors (CR)1 (CD35), CR2 (CD21), and the FDC marker FDC-M1 (Figure 7). (Bajénoff et al., 2006; Cremasco et al., 2014; Krishnamurty & Turley, 2020) MRCs

located between SCS and the outer edge of the B-cell zone are recognized by the expression of vascular cell adhesion molecule 1 (VCAM-1), ICAM-1, mucosal vascular addressin cell adhesion molecule 1 (MAdCAM-1), receptor activator of nuclear factor kappa-β ligand (RANKL), also known as tumour necrosis factor ligand superfamily member 11 (TNFSF11), and CCL19 (Figure 7). (Katakai et al., 2008) Moreover, upon lymphotoxin-β receptor (LTβR) stimulation, MRCs can secrete a large amount of CXCL13 to attract B-cells. (Suto et al., 2009) MRCs are also involved in the transportation of antigens, debris and DCs. Interestingly, MRCs can differentiate into FDCs upon inflammation in mice. (Jarjour et al., 2014)

### Pericytes

Pericytes are characterized by the expression of calponin-1, integrin-α 7 subunit (Itgα7), α-smooth muscle actin (αSMA) and are involved in smooth muscle contractility. (Link et al., 2007) Pericytes are positioned around the HEVs and together with perivascular FRCs and endothelial cells form a tight junction to ensheathe capillaries and maintain vascular integrity in both LNs and spleen (Figure 7). (Krishnamurty & Turley, 2020) Pericytes are also very similar to FRCs in their functional properties, such as the production of cytokines, chemokines and extracellular matrix components. (Krishnamurty & Turley, 2020)

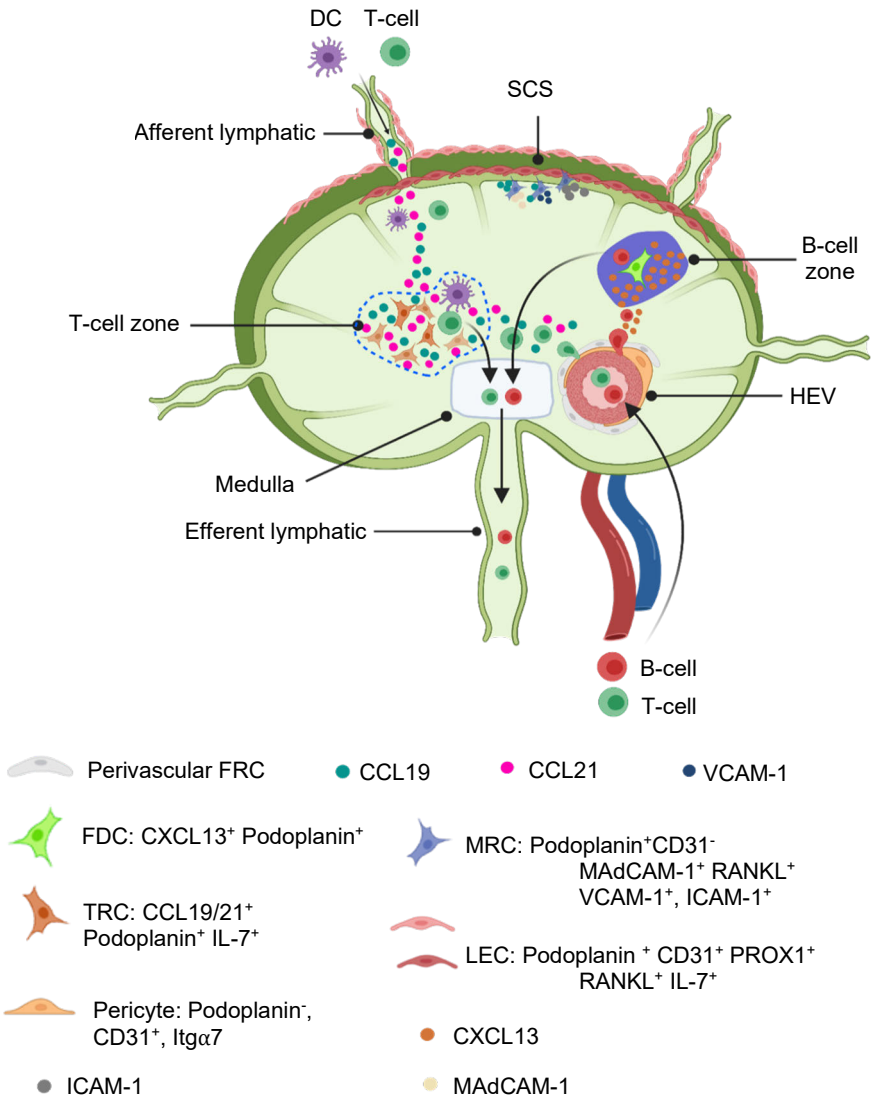


**Figure 7. The role of lymph node stromal cells in the homeostasis of leukocytes.** Dendritic cells (DCs) and a subset of T-cells that enters the lymph node from the afferent lymphatic vessels follow the gradient of CCL19 and CCL21 and enter the T cell zone. T- and B-cells can enter the lymph nodes from the high endothelial venules (HEVs). B-cells follow CXCL13 into the B-cell zone, whereas T-cells follow CCL19 and CCL21 expression into the T-cell zone. Abbreviations: subcapsular sinus (SCS), follicular dendritic cells (FDCs), T-cell zone FRCs (TRCs), integrin-α 7 (Itgα7), marginal reticular cells (MRCs), mucosal vascular addressin cell adhesion molecule 1 (MAdCAM-1), receptor activator of nuclear factor kappa-β ligand (RANKL), vascular cell adhesion molecule 1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), lymphatic endothelial cell (LEC), chemokine (C-X-C motif) ligand 13 (CXCL13), fibroblastic reticular cells (FRCs), chemokine (C-C motif) ligand 19 (CCL19), chemokine (C-C motif) ligand 21 (CCL21). Figure Modified from Krishnamurty et al. (Krishnamurty & Turley, 2020) This figure was created with www.biorender.com.

#### 2.2.2.1.2 Role of LNSCs in Immunity

LNSCs play a crucial role in maintaining tolerance in the LNs, where a balance between appropriate pro-inflammatory immune response against a pathogen and maintaining tolerance against self-reactive and commensal antigens is essential for a healthy immune system. Tolerance induction occurs in the LNs through deletion, anergy and $T_{reg}$ induction. $T_{reg}$ induction and deletion are the dominant mechanisms of tolerance induction in the thymus, whereas, deletion, anergy and $T_{reg}$ induction all occur in the LNs. Initially, immature LN-resident DCs without co-stimulatory molecules were thought to be the only leukocytes responsible for tolerance induction in the LNs. However, recently it has become clear that LECs and FRCs express antigens, including peripheral tissue antigens (PTAs), and can induce tolerance. (Mouchess & Anderson, 2013; Rouhani et al., 2015; Saxena et al., 2019) Interestingly, each subpopulation of LNSCs expresses distinct PTAs and secrete specific molecules to induce tolerance. (Cohen et al., 2010; Fletcher et al., 2010; Gardner et al., 2008)

##### Role of FRCs in Immunity

FRCs are among the most important nonhematopoietic stromal cells in LNs that are involved in regulating the adaptive immune response. FRCs are equipped with PRRs, such as TLR7, to detect intracellular single-stranded viral RNAs and can secrete inflammatory cytokines, such as TNF, IL-6 and CCL2. (Gil-Cruz et al., 2016) Among the FRCs subsets, FDCs were the first ones discovered to be able to present antigens. FDCs can capture and retain antigens, including antigen-antibody complexes through surface FC receptor expression, and present them to B cells. (Mandels et al., 1980) FDCs have also been shown to retain antigens for an extended time for presentation to LN-resident DCs. These DCs later can then present these antigens to $CD8^+$ T-cells and activate immune responses. (McCloskey et al., 2011) The location of FDCs also enables them to directly present antigens to antigen-specific B cells to initiate germinal centre reactions. (Qin et al., 1998; K. Suzuki et al., 2009; X. Wang et al., 2011)

FRCs also express MHC I and MHC II and can directly present self-peptides (Dubrot et al., 2014; J. W. Lee et al., 2007; Magnusson et al., 2008; Nichols et al., 2007) or viral peptides (Thomas et al., 2007) to naïve T-cells. However, the mechanism through which FRCs can acquire MHC complexes has been unknown until recently. Dubrot et al. showed that FRCs acquire the MHC II complex from the DCs to present it to naïve $CD4^+$ T-cells and induce dysfunctional self-antigen-specific T-cells. (Dubrot et al., 2014) MHC I expressed on FRCs is regulated by the transcription factor Deaf-1 to delete self-reactive $CD8^+$ T-cells. (Fletcher et al., 2010; Yip et al., 2009)

In addition to antigen presentation, FRCs use other inhibitory pathways to induce T-cell tolerance. For instance, in response to IFN-ɣ secreted by activated T-cells, FRCs produce a large quantity of inducible nitric oxide synthase (iNOS) to inhibit T-cell responses. Interestingly, the activity of Th2 cells, that do not secrete proinflammatory cytokines, are not inhibited by FDCs. (Khan et al., 2011) Another inhibitory pathway that FRCs use to induce T-cell tolerance is the programmed cell death protein 1 (PD-1) pathway. Upon inflammation, FRCs express high levels of programmed death-ligand 1 (PDL-1) and its interaction with its ligand, PD-1, suppresses the T-cell response (Mueller, Matloubian, et al., 2007).

## Role of LECs in Immunity

LECs can directly present an antigen to leukocytes or secrete different molecules to modulate the adaptive immune response. Like FRCs, LECs can also acquire PTAs from the lymphoid fluid and cross-present them to autoreactive $CD8^+$ T-cells via their MHC I molecules to induce peripheral tolerance. (Cohen et al., 2010; Tewalt, Cohen, Rouhani, Guidi, et al., 2012) These LEC-activated T-cells do not secrete effector cytokines and often undergo early apoptosis. (Dubrot et al., 2014; Hirosue et al., 2014)

In addition to presenting antigens, LECs can directly or indirectly modulate the maturation and function of DCs through direct contact or the secretion of different effector molecules. A recent study shows that culturing DCs with LEC-conditioned medium decreases the expression of MHC II, CD40 and IL-6 on DCs, while IL-10 and CCL2 expression were increased. Blocking the synthesis of prostaglandin by LECs partially recovers DC activation. LECs also contribute to the immune-inhibitory microenvironment by producing a large quantity of TGF-β1. (Christiansen et al., 2016) Moreover, LECs can directly modulate DC maturation and their functions by binding to MAC-1 on DCs via ICAM-1. In line with this, blocking the adhesive interaction between DCs and LECs by using anti-ICAM-1 blocking antibodies abolishes the immunosuppressive effect of LECs on DCs. (Podgrabinska et al., 2009) These data clearly show the immunosuppressive role of LECs on DCs and subsequently on the activation of the adaptive immune response.

In addition to tolerance induction at steady-state, it has recently been shown that LECs can scavenge tumour antigens and cross-present them to $CD8^+$ T-cells to induce activation and proliferation. (A. W. Lund et al., 2012) However, these LEC-activated $CD8^+$ T-cells in tumour-draining LNs express less INF-ɣ and bind more annexin V than hematopoietic-primed $CD8^+$ T-cells, suggesting a unique ability of LECs in suppressing tumour-specific $CD8^+$ T-cells by inducing apoptosis and inhibiting their cytotoxic functions. (A. W. Lund et al., 2012) Consistent with these

data, others have also shown that tumour-specific T-cells in tumour-draining LNs are exhausted in human melanoma. (Baitsch et al., 2011)

In addition to PTAs and tumour antigens, LECs can scavenge exogenous antigens and cross-present them to induce activation and proliferation of $CD8^+$ T-cells. (Hirosue et al., 2014) However, since LECs express high levels of MHC I and PD-1 but do not express co-stimulatory molecules, it was initially proposed that these LEC-activated and proliferating T-cells are dysfunctional or have an anergic phenotype at the steady-state. (Dubrot et al., 2014; Hirosue et al., 2014) However, recently, Vokali et al. showed that LEC-primed $CD8^+$ memory T-cells have the ability to reactivate and become effector T-cells in response to reoccurring inflammatory challenges, resulting in increased effector function and protective capacity. (Vokali et al., 2020) Others have also shown that unlike the tolerized $CD8^+$ T-cells that are primed by DCs and remain functionally impaired even in response to a pathogenic challenge, tolerized T-cells that are primed by liver sinusoidal endothelial cells (LSECs) can exit the non-responsive state and reactivate under pro-inflammatory conditions. (Derbinski et al., 2008; Villaseñor et al., 2008) LECs and FRCs also express MHC II at steady-state, and upon viral inflammation can generate dysfunctional $CD4^+$ T-cells and promote tolerance. (Dubrot et al., 2014; Malhotra et al., 2012; Ng et al., 2012) The endogenous basal expression of MHC II in LNSCs is controlled by promoter IV of class II transactivator (CIITA). In addition to the basal level, most MHC II expressed by LECs is acquired from DCs through direct contact with DCs or DC-derived exosomes. (Dubrot et al., 2014)

LECs can also effectively archive viral antigens for an extended period after the peak of the immune response to the antigen challenge. For instance, it has been shown that the flu-related antigens persists within LNs and can be expressed by LECs for more than 30 days after the initial infection. (Kim et al., 2010; Tamburini et al., 2014) Interestingly, the persistence of antigens on LECs is not dependent on lymphocytes, but rather on the proliferation of LECs during an efficient immune response. (Tamburini et al., 2014) Induction and persistence of LEC proliferation also requires inflammatory stimuli, such as TLR stimulation, along with vascular endothelial growth factor (VEGF) and IL-17 expression. (Aebischer et al., 2014; A. W. Lund et al., 2012; Tamburini et al., 2014) Although many studies have demonstrated the ability of LECs to acquire antigen at steady-state and during inflammation, the molecular mechanism through which LECs acquire and archive antigens for such a long time has remained largely unknown.

### 2.2.3 Spleen

The spleen is the largest organ of the lymphatic system. Like LNs, the spleen is home to many different immune cells. The spleen is the main reservoir of T-cells, memory

B-cells (Mamani-Matsuda et al., 2008), monocytes (Swirski FK et al., 2010; Van Der Laan et al., 2014) and platelets. (Badenhorst et al., 1985; Kotzé et al., 1986) Unlike the LNs, which are connected to the afferent lymphatic vessels filtering interstitial fluid from peripheral tissues, the spleen is connected to the blood circulation and filters the blood. The unique connection to the circulatory system enables the spleen to efficiently clear aged or infected red blood cells, soluble antigens, and particles from the blood. (Mebius & Kraal, 2005)

#### 2.2.3.1 Anatomy of the spleen

The unique architecture of the spleen and its vasculature enable this organ to filter a large quantity of blood and trap blood-borne antigens. The splenic vasculature can be imagined as arterial trees, in which small arterial branches end in a venous sinusoidal system. In rodents, the spleen consists of white pulp (WP), red pulp (RP) and marginal zone (MZ) (Figure 8). (Bronte & Pittet, 2013; Lewis et al., 2019; B. S. Steiniger, 2015) The anatomy of human spleen is very similar, however, the presence of MZ has remained controversial.

##### 2.2.3.1.1 Red Pulp

Red pulp consists of connective tissues and sinusoids filled with blood and immune cells. Immune cells in RP mainly consist of macrophages, neutrophils, monocytes, DCs, and a subset of T-cells known as γδ T-cells. (Nolte et al., 2000) One significant difference between the spleen and LNs is the lack of HEVs and afferent lymphatic vessels in the spleen. Instead, blood and newly-arrived immune cells can enter the spleen through the splenic vasculature.

The central arteries branch to small arteries that reach the RP to form the venous system. In rodents, some of these small arteries terminate in the MZ and form the MZ sinuses (Figure 8). RP arterioles are surrounded by $\alpha SMA^+$ smooth muscle and stromal cells. (Drenckhahn & Wagner, 1986; Groom et al., 1991; Nolte et al., 2000) (Drenckhahn & Wagner, 1986; Groom et al., 1991; Nolte et al., 2000) Arterial blood eventually ends up in the cords of the RP, which consists of reticular fibres and fibroblasts. These cords form an open blood system that lacks an endothelial lining. The blood then passes through the venous sinuses of the RP and eventually ends up in the efferent veins of the spleen (Figure 8). (Drenckhahn & Wagner, 1986; Groom et al., 1991) These venous sinuses lack continuous endothelial structures, and instead they have a discontinuous endothelium with stress fibres that extend underneath the basal plasma membrane and are arranged parallel to the cellular axis. (Drenckhahn & Wagner, 1986) The parallel structure of endothelial cells together with the arrangement of stress fibres in the sinuses forces the blood from the cords into the

sinuses by passing through the inter-endothelial slits (IES) that are formed by the stress fibres. (I. C. MacDonald et al., 1987) This passage is difficult for aged erythrocytes, as they have higher density and stiffer membranes. (Bratosin et al., 1998) The erythrocytes that are not able to pass the endothelium of sinuses stick in the cords and are phagocytosed by the cord macrophages. (Stewart & McKenzie, 2002) These cord macrophages also play an important role in recycling of iron that is acquired from the erythrocytes. (Knutson & Wessling-Resnick, 2003) The unique structure of the venous system also enables this area to uniquely filter and remove dead and opsonized cells from the blood circulation and simultaneously monitor the bloodstream for pathogens. (Nolte et al., 2000)

While the antigen specific immune responses takes place in the WP, most of the effector functions of immune cells take place in the RP. (Nolte et al., 2000) After their antigen-specific differentiation in the WP follicles, plasmablasts migrate to the RP to secrete antigen-specific antibodies. The location of these cells in the RP resembles the position of the plasmablasts in the medullary cords of the LNs, where extrafollicular antibodies are rapidly secreted to the lymph. (Johns & Christopher, 2012; Sze et al., 2000) The migration of plasmablasts into the RP depends on the upregulated expression of the chemokine receptor CXCR4 on the plasmablasts. This receptor binds to CXCL12, a chemokine that is produced by the stromal cells in the RP and LNs medullary cords. (Hargreaves et al., 2001) The upregulation of CXCR4 coincides with the downregulation of CXCR5 and CCR7 expression on B- and T-cells. These receptors bind to the homeostatic chemokines CXCL13 and CCL19/21, which are produced by the stromal cells in B-cell follicles and the T-cell zone, respectively, and anchor the cells in homeostasis. (García De Vinuesa et al., 1999)

The role of the RP is not only limited to its role in immunity. Under certain conditions, such as stress, RP has been shown to be a place for extramedullary hematopoiesis, i.e. hematopoiesis occurring outside of the medulla of the bone marrow. (Johns & Christopher, 2012) The initiation of extramedullary hematopoiesis is related to the ability of RP splenic stromal cells and perivascular stem cell niches to produce the essential factors for attraction, proliferation and differentiation of hematopoietic progenitors, such as stem cell factor (SCF) and CXCL12. (Inra et al., 2015; B. O. Zhou et al., 2014)

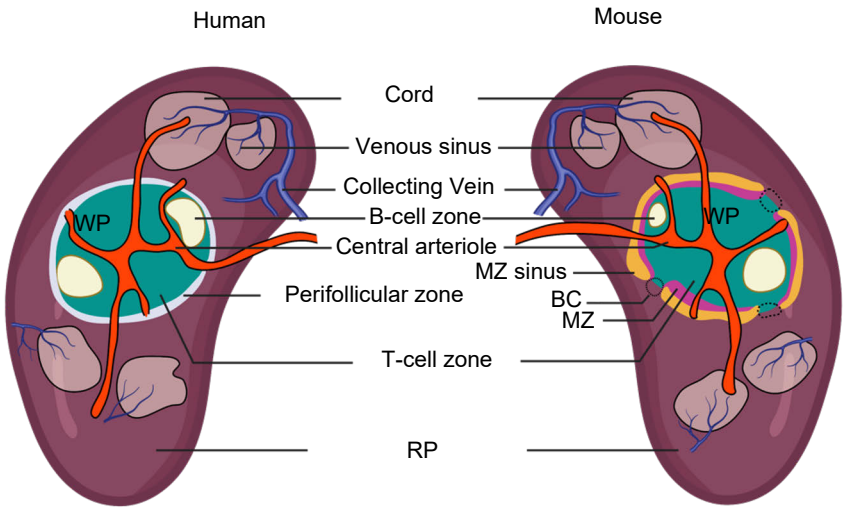


**Figure 8. Scheme of the human and mouse spleen.** The blood enters the spleen via the central arterioles. The central arterioles are sheathed by the white pulps. White pulp (WP) consists of B- and T-cell zones. The central arterioles branch out into small arteries that traverse the marginal zone (MZ) and enter the red pulp (RP) to form the venous system of the red pulp. Bridging channels (BCs) connect the RP to WP. In mice, some of these small arteries end in the MZ sinuses. Human spleen does not have the MZ sinus and instead it contains a perifollicular zone, where the bloodstream is partially released. Therefore, one can envisage that the perifollicular zone plays a similar role to marginal zone sinuses. Blood eventually enters the RP cords that consist of fibroblast and reticular fibres. These cords are home to many macrophages. Blood eventually returns to the blood circulation via the collecting veins. This figure was created with www.biorender.com.

#### 2.2.3.1.2 White pulp (WP)

The spleen consists of multiple LN-like structures known as the WP. Like LNs, splenic WP consists of a T-cell zone and B-cell follicles. Unlike LNs, WP is not surrounded by a capsule to separate it from the splenic parenchyma. Instead, in rodents, it is surrounded by a rim of innate immune cells forming the MZ (Figure 8). (Mebius & Kraal, 2005) In addition to MZ, one of the fundamental differences between LNs and WP is their mode of antigen collection. Unlike LNs, which are embedded in a network of lymphatic vessels through which antigens arrive from peripheral tissues, the spleen is located in the centre of the blood circulation and directly receives blood borne-antigens from across the body. (Nolte et al., 2003)

In humans and mice, blood first enters the spleen via the splenic arteries. Splenic arteries later branch out into the trabeculae of the spleen, where they are called trabecular arteries and are surrounded by a dense connective tissue originating from the spleen capsule. Trabecular arteries eventually spread out into the T-cell zone in the WP and form the central arteries. Since the T-cell zones are formed around the

central artery, these structures are also called periarterial lymphatic sheaths (PALS). PALS are attached to B-cells follicles in mice and consists of FRCs, macrophages, DCs and T-cells. B-cells follicles are mainly populated by B-cells (Figure 9). Similar to LNs, upon an invading pathogen, naïve and memory T-cells become activated and initiate the GC B-cell reaction to induce the production of antigen-specific antibodies in a T-dependent manner. (Pereira et al., 2010)

Stromal cells in the splenic WP consist of FRCs of the T-cell zone, FDCs of the B-cell zone and marginal reticular cells situated in the outer region of the follicles. (den Haan et al., 2012) The splenic conduit system forms a network of collagen that is ensheathed by FRCs. (Nolte et al., 2003) In mice, these FRCs form a highway that penetrates the MZ via the marginal zone bridging channels (MZ-BCs) and connects the PALS to the RP. (Nolte et al., 2003) Initially, it was believed that the conduit system is not actively involved in cell migration and antigen transfer into the WP. However, recently it has been shown that the splenic conduit system regulates the exchange of molecules with the bloodstream. For instance, small blood-borne molecules, such as cytokines and antigens, can enter the WP, while large molecules with high molecular weight, such as immunoglobulin G (150 kDa) and Dextran (500 kDa), cannot access the WP. (Nolte et al., 2003)

WP stromal cells also play an important role in homeostasis. FRCs and FDCs are the source of CCL21 and CXCL13, respectively, through which they selectively guide lymphocytes into different compartments of the WP (Figure 9). (Nolte et al., 2003) The migration of B-cells into the splenic follicles highly depends on CXCL13. CXCL13-deficient FDCs fail to guide B-cells into the splenic follicular region. (Ansel et al., 2000) The number of T-cells in the splenic T-cell zone highly depends on the expression of both CCR7 on T-cells and its ligands CCL19/21. Mice lacking CCR7 have scattered T-cells throughout their spleen. (Acton et al., 2012; Calabro et al., 2016; Förster et al., 1999; Gunn et al., 1999; Nolte et al., 2003) DCs also use CCL19/21 to find their way to the WP and to adhere to FRCs, which allows them to meet the newly arrived naïve T-cells that enter the WP (Mori et al., 2001). Similar to FDCs in the LNs, FDCs in the spleen express ICAM-1, complement receptors (Allen et al., 2007), as well as low levels of VCAM-1 and MADCAM-1 at steady-state. (Balogh et al., 2002; Ree et al., 1993) Once insulted, FDCs upregulate adhesion molecules and complement receptors, such as ICAM-1, VCAM-1, CD21, CD35 and Fc-receptors, which play an essential role in forming the GC and activating the B-cells. (Allen et al., 2007; Balogh et al., 2002; Maeda et al., 1995) It has been implicated that T-cells also use the same molecules to migrate into the WP. (Lu & Cyster, 2002)

#### 2.2.3.1.3 Marginal Zone (MZ)

In mice, MZ surrounds the WP and separates the RP from the WP. MZ is populated by different immune cells, including B-cells, DCs and macrophages. (Lu & Cyster, 2002) There are two main subsets of macrophages anchored by the CCL19/21 chemokine signals in the MZ (Ato et al., 2004) : (i) The marginal zone macrophages (MZMs), and (ii) the marginal zone metallophilic macrophages (MMMs) (Figure 9). These macrophages play an important role in capturing non-opsonized molecules and blood-borne antigens. (Elomaa et al., 1995; Geijtenbeek et al., 2002; Kraal & Janse, 1986) The MZMs form the outer ring of macrophages and are closer to the RP. These macrophages can be characterized by the expression of macrophage receptor with collagenous structure (MARCO) (Elomaa et al., 1995) and C-type lectin specific intercellular adhesion molecule-3-grabbing nonintegrin-related 1 (SIGN-R1). (Geijtenbeek et al., 2002; Kang et al., 2003, 2004) The MMMs form the inner ring of macrophages and are closer to the WP. These macrophages can be characterized by the expression of sialic acid binding Ig like lectin 1 (SIGLEC-1). (Munday et al., 1999) The MZ sinuses are located between these two subsets of macrophages and are formed by nonhematopoietic sinus lining cells (Figure 9). These sinus-lining cells can be characterized by the expression of MADCAM-1. (Girkontaite et al., 2004) A subset of DCs and marginal zone B-cells are also located between these two subsets of macrophages (Figure 9). (Qiu et al., 2009) In contrast to mice, the human spleen does not have MZ sinuses, and instead, the perifollicular zone is situated between the follicular MZ and the RP (Figure 8). (B. Steiniger et al., 2001)

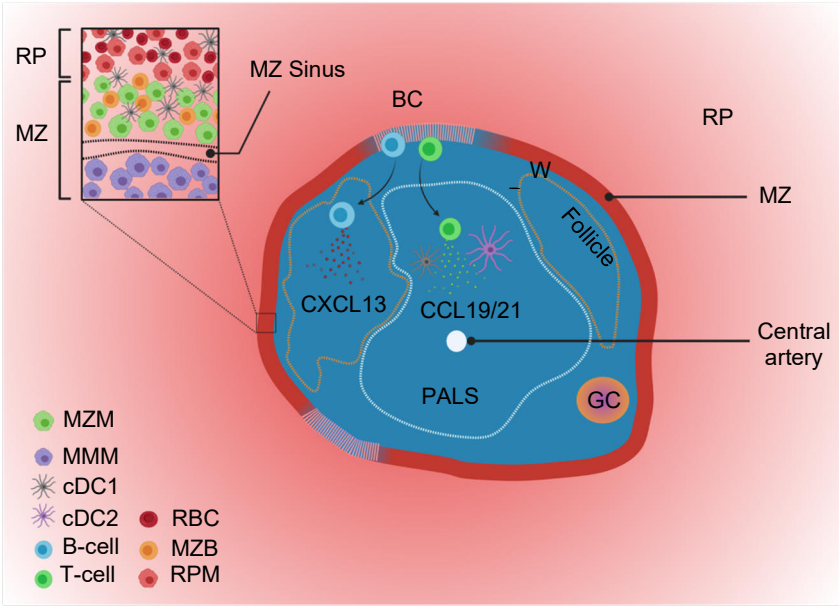


**Figure 9. Structure of the normal spleen in mice.** Marginal Zone (MZ) separates the white pulp (WP) from the red pulp (RP). The WP consists of B-cell follicles and Periarterial lymphatic sheath (PALS). PALS consists of the T-cell zone and DCs. Bridging channels (BCs) penetrate the MZ and connect the WP to RP. MZ consists of marginal zone macrophages (MZM), marginal zone metallophilic macrophages (MMM), marginal zone B-cells (MZBs), conventional dendritic cells 1 (cDC1) and the MZ sinus. Stromal cells within the follicles and T-cell zone express CXCL13 and CCL19/21, respectively. Abbreviations: red blood cell (RBC), RP macrophages (RPMs), conventional dendritic cells 2 (cDC2), chemokine (C-X-C motif) ligand 13 (CXCL13), chemokine (C-C motif) ligand 19 (CCL19), chemokine (C-C motif) ligand 21 (CCL21), germinal centre (GC). Figure adapted from Lewis et al., 2019. (Lewis et al., 2019) This figure was created with www.biorender.com.

The marginal zone B-cells (MZBs) play a central role in the organization and integrity of the MZ. The absence of B-cells from birth or their depletion after birth results in the disappearance of both subsets of MZ macrophages. (Crowley et al., 1999; Nolte, Arens, et al., 2004) B-cells also play an important role in the upregulation of CXCL13 by stromal cells and, in turn, the recruitment of CR-Fc$^{+}$ DCs to the MZ and B-cell follicles. (Yu et al., 2002) Interestingly, the expression of MARCO by the marginal zone macrophages is essential for the retention of marginal zone B-cells. (Karlsson et al., 2003)

MZBs are anchored through their LFA-1 and α4β7 integrins bound to ICAM-1 and VCAM-1, respectively. (Lu & Cyster, 2002) In addition, MZBs express high levels of sphingosine 1-phosphate (S1P) receptor 1 and 3 ($S1P_1$ and $S1P_3$, respectively), which have been shown to be required for B-cell retention in the MZ. In contrast to MZBs, follicular B-cells express low levels of S1P receptors.

(Cinamon et al., 2004; Girkontaite et al., 2004) In mice lacking $S1P_1$, but not $S1P_3$, B-cells fail to lodge in the MZ. In mice lacking CXCL13, $S1P_1$-deficient B-cells regain their ability to localize in MZ. These findings suggest that $S1P_1$ signalling dominate the recruiting activity of CXCL13 and thus retain the B-cells in the MZ. Consistent with this, B-cells rapidly downregulate their expression of $S1P_1$ as well as $S1P_3$ and migrate to the B-cell follicles in the WP after LPS treatment or antigen exposure. (Cinamon et al., 2004)

Upon encountering an antigen, MZBs capture the antigen and migrate to WP to interact with T- and B-cells to initiate the adaptive immune response. (Arnon et al., 2013) An elegant intravital microscopy study showed that B-cells constantly shuttle between the B-cell zone in the WP and MZ, suggesting a critical role of B-cells in transferring blood-borne antigens into the WP. (Arnon et al., 2013) In addition to B-cells, DCs also reside in MZ, capable of capturing and transferring antigens to the T-cell zone of WP. (Qiu et al., 2009) In an elegant study, Calabro and colleagues showed that two distinct subsets of DCs with different functionality reside in MZ: (i) $33D1^+$ DCs that migrate to the $CD4^+$ T-cell zone, and (ii) $XCR1^+$ DCs that migrate to the $CD8^+$ T-cell area of the WP. (Calabro et al., 2016)

In addition to containing many resident cells, the MZ is an important transit area for immune cells that are leaving the bloodstream and entering the WP. The exact molecular mechanism of the transiting cells has remained largely unknown. Almost 60 years ago, it was demonstrated that intravenously injected India ink primarily accumulates in MZ, and at multiple locations WP penetrates the MZ rim and enters the RP. Similarly, intravenous injection of labelled lymphocytes shows multiple channels of lymphocytes penetrating the MZ, i.e. structures called bridging channels (BCs), also known as MZ-BCs (Figure 9). MZ-BCs are identified as a rich FRC network that penetrates the MZ and connects the PALS to RP. How these MZ-BCs contribute to lymphocyte trafficking within the spleen has remained largely controversial. Initial studies favoured a model that lymphocytes use the MZ-BCs as an exit route out of the WP, through which they can directly access venous sinuses in the RP and return to the blood circulation. (Brelińska et al., 1984; Mitchell, 1973) In contrast, recently, a sequential imaging study suggested that after entering the spleen parenchyma via the MZ sinuses, lymphocytes use the MZ-BCs to enter the WP. (Bajénoff et al., 2008) These findings led to the conclusion that lymphocytes first enter the spleen parenchyma via the MZ sinuses, and then use the MZ-BCs as a corridor to bidirectionally migrate between the WP and RP. More recently, an elegant intravital microscopy study showed that, after entering the parenchyma, lymphocytes use the MZ-BCs to unidirectionally migrate to the WP. (Chauveau et al., 2020)

#### 2.2.3.2 The role of the spleen in immunity

After entering the spleen parenchyma, blood passes through the MZ and enters the WP. Its unique structure and location enable the spleen to efficiently monitor the blood for blood-borne antigens. Both the adaptive and innate immune responses can be initiated in the spleen. While the WP is mostly involved in mounting the adaptive immune response, the MZ is involved in both innate and adaptive immune responses. The effector function of immune cells primarily takes place in the RP.

The overall organization and immune cell composition of the spleen are very similar to that in the LNs. One important difference is the distribution of APCs between WP and LNs. In the spleen, APCs are strategically located in the MZ, where they can efficiently detect blood-borne antigens (Nolte et al., 2000). These cells express a wide range of specific receptors, including PRRs, that enable them to efficiently capture antigens. Some of these receptors are unique to this region. For instance, macrophages in the MZ express MARCO and SIGNR1. MARCO can recognize a wide range of pathogens, such as *Staphylococcus aureus* and *Escherichia coli*. SIGNR1 can efficiently bind to polysaccharide antigens, such as mannosylated lipoarabinomannan, which are present on the surface of *Mycobacterium tuberculosis.* (Kang et al., 2003; Koppel et al., 2004) SIGNR1 also mediates the uptake and clearance of *Streptococcus pneumoniae.* (Kang et al., 2004; Koppel et al., 2004) After binding, macrophages internalize and target *M. tuberculosis* to lysosomes for degradation. (Geijtenbeek et al., 2002) In addition to bacterial infections, marginal zone macrophages are also able to capture and clear viral infections (Oehen et al., 2002). Upon ingesting a virus, MMMs secrete a large quantity of IFN-α and IFN-β to initiate the immune response, while MZMs express a lesser amount of these cytokines. (Eloranta & Alm, 1999) On the other hand, the MZMs can ingest a large quantity of all particles that enter the spleen, digest them in their lysosomes and shed the antigenic fragments to activate the MZBs. (Van Rooijen, 1990)

Resident B-cells in the MZ are also able to recognize blood-borne antigens. Upon activation, they either differentiate into IgM-producing plasma cells or migrate to the follicles in the WP to present the captured antigens to other immune cells. (Lopes-Carvalho & Kearney, 2004; Oliver et al., 1999) The migration of B-cells into the follicles is mediated by the downregulation of $S_1P_1$ and $S_1P_3$, which enables them to respond to CXCL13 expressed by FDCs in the follicles. (Cinamon et al., 2004)

Another important subpopulation of immune cells in the MZ are DCs, which play a crucial role in immunity. Upon activation, marginal zone DCs migrate into the WP to activate the adaptive immune response. This migration is crucial for the activation of the adaptive immune response, as shown during chronic infection with *Leishmania donovani* where DCs downregulate their expression of CCR7 and lose their ability to migrate to WP, resulting in spreading the infection (Ato et al., 2002).

Activated B-cells also play a central role in the migration of CR-FC$^+$ DCs into the follicles and subsequent initiation of the adaptive immune response. (Yu et al., 2002)

Once APCs activate the T-cells in the WP, CD4$^+$ T-cells downregulate their expression of CCR7 while upregulating the expression of CXCR5. This enables them to relocalize to the T-cell/B-cell border, where they can interact with B-cells. (Ansel et al., 1999; Fillatreau & Gray, 2003) Similarly, upon activation, B-cells upregulate their expression of CCR7 and migrate to the T-cell/B-cell border, where they can receive help from the activated CD4$^+$ T-cells and undergo isotype switching. (Reif et al., 2002) After this, activated B-cells either migrate to the MZ and RP or stay within the germinal centre. (Pape et al., 2003) Accordingly, the importance of the spleen in mounting a proper antibody response against thymus-independent antigens, such as several bacterial products, has been demonstrated in both splenectomised patients and mice (Amlot & Hayes, 1985).

In addition to spleen-resident immune cells, blood immune cells can also capture antigens and migrate to the spleen. For instance, in the initial response to bacterial infection, blood DCs are one of the first subsets of immune cells that capture and transport the bacterial components to the spleen. In the spleen, these DCs promote B-cell differentiation into antibody-producing plasmablasts. This occurs in the T-B border and bridging channels. (Balázs et al., 2002).

### 2.2.4 Clever-1, also known as FEEL-1 and Stabilin-1

In 2003, a novel adhesion molecule, now known as common lymphatic endothelial and vascular endothelial receptor-1 (Clever-1), also called Stabilin-1 and FEEL-1, was identified by two groups simultaneously. (Irjala, Johansson, et al., 2003; Politz et al., 2002) Irjala et al. identified Clever-1 by producing two different mAbs, called 3-266 and 3-72, against human LECs. The antibodies strongly stained vascular and lymphatic endothelium, including HEVs and both afferent and efferent lymphatic vessels, but not the immune cells. Simultaneously, Politz and colleagues identified Clever-1 as the antigen recognized by the MS-1 antibody and they called it Stabilin-1. This antigen was expressed by sinusoidal endothelial cells of the human spleen and a subset of macrophages. (Politz et al., 2002) At the same time, another group, led by Adachi and Tsujimoto, cloned a scavenger receptor (Clever-1), and since the molecule consisted of 7 fasciclin (Fas-1), 16 epidermal growth factor (EGF)-like, 2 laminin-type EGF-like domains and one link domain, they named it FEEL-1 (Figure 10). (Adachi & Tsujimoto, 2002) Analysis of the cDNA of Clever-1 showed it to be a 270-280 kDa type I transmembrane protein, which contains 69 exons and has at least two isoforms at the protein level. (Irjala, Johansson, et al., 2003)

Clever-1 is functionally classified as a class H scavenger receptor and is a close relative of another adhesion molecule, called Stabilin-2, with 55% similarity at the

protein level despite no significant DNA homology. (Kzhyshkowska et al., 2004; Politz et al., 2002) Despite these similarities, their expression pattern, ligand repertoire and subcellular localisation are different. Stabilin-2 was initially identified by hyaluronan chromatography and has two main isoforms: 173/300 kDa and 190/315 kDa. (Harris et al., 2007) Stabilin-2 is expressed on sinusoidal endothelial cells of the liver, spleen, bone marrow and LNs. (Falkowski et al., 2003) Stabilin-2 binds to a wide range of ligands, including procollagen peptides and advanced glycation end-product (AGE)-modified proteins. (Kzhyshkowska et al., 2004) Stabilin-2 is involved in clearing different unwanted products, such as necrotic cells. (D'Souza et al., 2013) Moreover, it is an endocytic receptor for HA, responsible for systemic HA clearance. (Harris et al., 2008; B. Zhou et al., 2000; Bin Zhou et al., 2002, 2003) Stabilin-2 is also involved in lymphocyte binding to the endothelial cells in the liver. (Jung et al., 2007) In contrast, Clever-1 does not bind to HA, despite containing an X-link domain, and has a lower affinity to AGE-modified proteins (Kzhyshkowska et al., 2004). Collectively, these findings suggest a possible role of Clever-1 as a scavenger receptor in internalizing and clearing unwanted molecules, such as LDL.

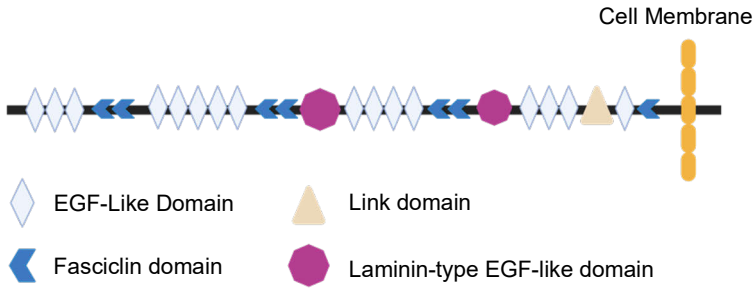


**Figure 10.** Schematic structure of Clever-1. Adapted from Adachi et al. (Adachi & Tsujimoto, 2002). This figure was created with www.biorender.com.

### 2.2.4.1 Expression pattern of Clever-1

In humans, Clever-1 is expressed by sinusoidal endothelial cells in the liver, spleen, and bone marrow, on HEVs of the LNs and tonsils, as well as afferent and efferent lymphatic endothelial cells. (Politz et al., 2002; Qian et al., 2009; Salmi et al., 2004) In addition, Clever-1 is also expressed by microglia, peritoneal macrophages, adipose tissue macrophages, type II macrophages (M2) and tumour-associated macrophages (TAMs). (Ålgars et al., 2012; Boström et al., 2015; Karikoski et al., 2014; Palani et al., 2011; Viitala et al., 2019) However, the expression pattern of Clever-1 varies between human and mouse. For instance, mouse HEVs express very low levels of Clever-1 or lack it completely when analysed with confocal microscopy, while human HEVs express high levels of Clever-1. (Karikoski et al., 2009)

Clever-1 constantly cycles between the endosomal compartments and plasma membrane. (Prevo et al., 2004) Upon inflammation, Clever-1 is upregulated on the blood endothelium of inflamed tissues, such as tissues undergoing wound healing, skin psoriasis and endothelium of melanoma metastasis. (Salmi et al., 2004) Clever-1 is primarily localized in early or recycling endosomes and the trans-Golgi network (TGN), while it rarely is present in $CD63^+$ lysosomes and late endosomes. (Kzhyshkowska et al., 2004) Interestingly, treating macrophages with Brefeldin A, a Golgi inhibitor, results in redistribution and accumulation of Clever-1 mainly in the TGN. (Kzhyshkowska et al., 2004) It has been also shown that inflammation can change the expression pattern of Clever-1 from the cell surface to inside the cells. (Kzhyshkowska et al., 2004; Palani et al., 2016; Prevo et al., 2004)

#### 2.2.4.2 Role of Clever-1 in homeostasis

Different biological macromolecules, such as collagen, lipids, nucleic acids, and proteins, are generated during physiological cell turnover and tissue remodelling. These products either accumulate locally or may spill over into the bloodstream. For instance, extracellular matrix degradation products, including collagens or glycosaminoglycans, are released into the bloodstream, and lymph and their accumulation in the body can be detrimental. Thus, a systemic clearance receptor is vital to remove these products from the body to maintain homeostasis.

Sinusoidal endothelial cells and macrophages are both considered as professional scavengers. Sinusoidal cells scavenge unwanted molecules from the blood and lymph, and macrophages clear the unwanted products from the extracellular spaces in tissues. Both sinusoidal endothelial cells and M2 macrophages express high levels of Clever-1. Clever-1 can bind to secreted protein acidic and rich in cysteine (SPARC) (Kzhyshkowska, Workman, et al., 2006), placental lactogen (Kzhyshkowska et al., 2008), Stabilin-1-interacting chitinase-like protein (SI-CLP) (Kzhyshkowska, Mamidi, et al., 2006), acetylated low-density lipoprotein (Ac-LDL), bacteria, and apoptotic cells, and function as a scavenger receptor. (D'Souza et al., 2013; Kzhyshkowska et al., 2004, 2008; Kzhyshkowska, Workman, et al., 2006; Park et al., 2009; Y. Tamura et al., 2003) SPARC and placental lactogen are efficiently endocytosed by Clever-1 expressed by M2 macrophages and then partially degraded in lysosomes. (Kzhyshkowska et al., 2008; Kzhyshkowska, Workman, et al., 2006) The undegraded placental lactogen is later transferred to TGN, dissociated from Clever-1 and stored in storage vesicles. (Kzhyshkowska et al., 2008) In the case of SI-CLP, Clever-1 transports it from early/sorting endosomes to the TGN and eventually sorts it into the late endosomes and secretory lysosomes. (Kzhyshkowska, Mamidi, et al., 2006)

Mice lacking Clever-1 and Stabilin-2 develop perisinusoidal fibrosis in their liver, suggesting a role for these molecules in the hepatic clearing of noxious agents.

(Schledzewski et al., 2011) It also has been shown that Clever-1$^+$ macrophages rapidly accumulate around the inflamed tissue in a mouse model of liver fibrosis and human liver diseases, and mice lacking Clever-1 develop more severe and persistent hepatic fibrosis than WT mice, suggesting a possible role of Clever-1$^+$ macrophages in the resolution of liver injuries. (Rantakari et al., 2016) Moreover, M2 Clever-1$^+$ macrophages can bind, internalize and degrade placental lactogen. (Kzhyshkowska et al., 2008)

In addition to its scavenging functions, Clever-1 is associated with an adhesive function and plays an essential role in leukocyte migration into different tissues. The early *ex vivo* studies show that Clever-1 on blood and lymphatic endothelial cells mediates the rolling and transmigration of immune cells. (Salmi et al., 2004; Shetty et al., 2011) More recent studies have shown that human placental macrophages express high levels of Clever-1, and it regulates their adhesion to placental vascular endothelium and mediates their transmigration. (Palani et al., 2011) In addition to adhesion, Clever-1 regulates the intracellular crawling of lymphocytes through the hepatic sinusoidal endothelial cells. (Patten et al., 2017) Clever-1 is also reported to be upregulated on the inflamed human hepatic endothelial cells, supporting T-cell migration to the inflamed liver. (Shetty et al., 2011) Despite the undeniable role of Clever-1 in leukocyte migration, its ligand on the leukocytes has not been identified.

#### 2.2.4.3 Role of Clever-1 in Immunity

Clever-1 is considered as an immunosuppressive molecule whose expression on macrophages and monocytes has been associated with their anti-inflammatory phenotype and immunosuppressive conditions (Palani et al., 2011, 2016). Clever-1 expression is upregulated on macrophages treated with dexamethasone and IL-4, while inhibited when treated with IFN-ɣ. (Goerdt et al., 1993; Kzhyshkowska et al., 2004) Consistently, *ex vivo* studies demonstrate that macrophages deficient in Clever-1 express more TNF-α, and once co-cultured with B-cells, enhance antibody responses in B-cells. (Dunkel et al., 2018) Recently, Palani et al. showed that Clever-1 expression on monocytes is downregulated under pro-inflammatory conditions, while it is upregulated in the immunosuppressive microenvironment and Th2 response. Furthermore, they demonstrated that in pregnant women with pre-eclampsia, which is associated with abnormal pro-inflammatory reactions, Clever-1 expression on CD14$^+$ blood monocytes is significantly lower than in healthy pregnant women, suggesting a correlation between the expression level of Clever-1 and an immunosuppressive microenvironment. (Palani et al., 2016) Similarly, Clever-1 supports the transmigration of immunosuppressive CD4$^+$FOXP3$^+$ Tregs across the human hepatic sinusoidal endothelial cells (HSECs). (Shetty et al., 2011) Collectively, these observations strongly support the function of Clever-1 as an immunosuppressive molecule in different cells in addition to its scavenging and adhesive functions.

# 3 Aims

Leukocyte migration into secondary lymphoid organs plays a fundamental role in the efficient activation of immune responses. Many molecules have been shown to mediate the migration of leukocytes to LNs. Yet, the molecular mechanism of lymphocyte migration into the spleen is largely unknown. In addition, only a few molecular interactions have been shown to mediate both the migration of leukocytes and the magnitude of antigen-specific immune response in the LNs. We performed these studies to further elucidate the role of Clever-1 in leukocyte trafficking into secondary lymphoid organs as well as its regulatory function on eliciting antigen-specific immune responses. The specific aims of this thesis work were:

1- Investigating the expression pattern of Clever-1 on spleen vasculature and its role in the migration of lymphocytes into the spleen.

2- Studying the expression pattern of Clever-1 on peripheral lymphatic vessels and its regulatory role on DC migration and T-cell activation in the dLNs.

# 4 Material and Methods

## 4.1 Animals (I & II)

Animals used in both publications of this thesis work were housed in the animal facilities of Turku University (Turku, Finland). Animals were kept in individual-ventilated cages at 22°C with a 12-hour light-dark cycle. Mice were fed with dried chow pellets and water. Both genders, male and female, from 6- to 8- week-old, were used in this thesis work. All animal experiments were reviewed and performed according to the rules and regulations of The Finnish Act on Animal Experimentation (62/2006) with respect to the 3Rs principle. The local committee approved animal experiments for animal experimentation under the following animal licence numbers: 5587/04.10.07/2014, ESAVI/5762/04.10.07/2017, 5762/04.10.07/2017 and 12537/2020.

Clever-1 knockout (KO) mice with mixed C57BL/6N or 129SvJ background were generated as described (Karikoski et al., 2014). Wild-type (WT) mice with mixed C57BL/6N or 129SvJ background were used as controls for the KO mice. C57BL/6N and $CD11c^{+}$-$YFP^{+}$ reporter mice were purchased from the Jackson Laboratory. Balb/c mice were purchased from Janvier and Charles River. KikGR transgenic mice (i.e. so-called Kikume mice), which express a Kikume Green-Red photoconvertible fluorescent protein, (Tomura et al., 2014) were a generous gift from Professor Masayuki Miyasaka, Osaka University. OT II mice, which express α- and β-chain T-cell receptors specific for chicken ovalbumin (OVA), were purchased from Jackson laboratory. DsRed mice [B6.Cg-Tg(CAG-DsRed*MST)1Nagy/J] carrying a red fluorescent protein (DsRed) were purchased from the Jackson Laboratory (stock number 006051). CD45.1 allele–carrying B6.SJL-PtprcaPepcb/BoyJ mice were purchased from the Jackson Laboratory, and C57BL/6JRj WT mice carrying the CD45.2 allele were purchased from Janvier.

### 4.1.1 In vivo experiments

#### 4.1.1.1 Lymphocyte homing into the spleen (I)

Lymphocytes were obtained by mechanical disruption of the spleen of WT donor mice. The lymphocytes were labelled with 5 µM of CFSE (Molecular probes), according to the manufacturer's instructions. The CFSE-labelled lymphocytes were injected intravenously (i.v.) into the tail vein of KO and WT recipient mice. After 2 hours, the spleen, blood, peripheral and mesenteric lymph nodes were collected, and single cell suspensions were prepared. The single cells were stained with CD4, CD8, and B220 antibodies listed in Table 3. The number of transferred lymphocytes was analysed with flow cytometry.

In another set of experiments, lymphocytes were isolated from the spleen of WT donor mice and stained with 5 µM of Vybrant CFDA SE cell tracer (CFSE; Thermo Fisher Scientific). 30 million cells in 200µl of phosphate-buffered saline (PBS) were injected i.v. into the tail vein of recipient KO and WT recipient mice. Mice were sacrificed 10 minutes or 2 hours later and their spleen was collected. The number of transferred cells in RP, WP and MZ of the spleen was counted with fluorescence imaging.

Alternatively, to identify the entry site of lymphocytes and their distribution within the spleen, lymphocytes were harvested from the spleen of $CD45.1^+$ donor mice and labelled with 5 µM Vybrant CFDA SE cell tracer (CFSE; Thermo Fisher Scientific). The CFSE-labelled cells were injected i.v. into $CD45.2^+$ recipient mice. Mice were sacrificed 10, 30 and 120 minutes later and their spleens were collected. The location of transferred cells was analysed with fluorescence imaging using an antibody against CD45.1 (Table 3). Alternately, to avoid extra artefacts caused by CFSE labelling, 10 million $DsRed^+$ lymphocytes in 100 µl PBS were isolated from the spleen of DsRed transgenic mice and injected i.v. into KO and WT recipient mice through their tail veins. The location of transferred cells was analysed with fluorescence imaging. In this set of experiments, MAdCAM-1 staining was used to identify the MZ. The whole section in a total area of $0.64mm^2$ was imaged, and the number of cells located within different regions of the spleen was quantified using ImageJ. The number of cells in each region were normalized to the area of the region.

#### 4.1.1.2 Ovalbumin-specific T-cell response (II)

To evaluate the antigen specific T-cell response, the LPS-matured WT Bone marrow derived-DCs (BMDCs) were generated and loaded with 2.5µg of $OVA_{323-339}$ peptide (SSINFEKL) and then washed vigorously with PBS. $5.5 \times 10^4$ cells were injected intradermally into the footpad of KO and WT recipient mice. One day later,

lymphocytes were isolated from the spleen of OT II mice and labelled with 5 µM Vybrant CFDA SE cell tracer (CFSE; Thermo Fisher Scientific). 5 ×10 $^{6}$ cells in 200µl of PBS were injected i.v. into the tail vein of KO and WT recipient mice. After 48 hours, mice were sacrificed and their popliteal dLNs were collected. Single-cell suspensions were prepared and cells were stained with CD45, CD3, B220, and CD4 antibodies listed in Table 3. The number of transferred cells and their proliferation were analysed with flow cytometry.

#### 4.1.1.3 Contact Hypersensitivity Model (CHS) (II)

To induce the CHS model, KO and WT mice were first sensitized by applying 2% OXA ((4-ethoxymethylene-2-phenyl-2- oxazoline-5-one; Sigma-Aldrich) in acetone/olive oil (4:1 volume/volume) on their shaved belly (50 µl) and paws (5 µl). Five days later, the mice were challenged by applying 10 µl of 1% OXA solution on both the dorsal and ventral sides of the ears. The thickness of the inflamed ears was measured from day 1 to day 8 after the OXA challenge. Normal non-inflamed ears were used as a control.

In CHS adoptive transfer experiments, to generate OAX-primed T-cells, DsRed transgenic donor mice were sensitized with 2% OXA. Five days later, the skin-draining axillary and inguinal LNs were collected and a single-cell suspension was prepared. 1×10$^{7}$ cells in 160 µl of PBS were injected i.v. into the tail vein of KO and WT mice. Alternatively, T-cells were negatively isolated using a T-cell isolation kit (EasySep$^{TM}$). 1×10$^{7}$ cells in 160 µl of PBS were injected i.v. into the tail vein of KO and WT mice. One day later, mice were challenged by applying a 1% OXA solution on both the dorsal and ventral sides of the ears. The thickness of the inflamed ears was measured one and two days after the OXA challenge.

For evaluating the leukocyte compositions in the non-inflamed and inflamed ears, mice were sacrificed and single cells were isolated from the ear skin of KO and WT mice, using whole skin dissociation kit (Miltenyi Biotec) according to the manufacturer's instructions. The single-cell suspensions were stained with antibodies against CD45, CD3, CD8a, CD4, MHC II, CD11c, CD103, CD25 and CD11b (Table 3). To quantify the Langerhans cells, samples were fixed with 4% paraformaldehyde, washed and permeabilized using permeabilizing buffer (eBioscience), and stained with a Langerin-Alexa Fluor 647 antibody (Dendritics). To quantify the number of Foxp3 cells, single-cell suspensions were fixed and permeabilized using Transcription Factor Staining Buffer Set (eBioscience) according to the manufacturer's instructions. Samples were then stained with Foxp3-PE-CF594. The number of leukocytes was analysed by flow cytometry.

#### 4.1.1.4 Induction of lymphopenia and blockade of lymphocyte exit (I)

For evaluating the entry of lymphocytes into lymphoid organs in the absence of Clever-1, KO and WT mice were treated with polyinosinic-polycytidylic acid [poly(I:C)] (Sigma). Poly I:C is a synthetic analogue of double-stranded RNA present in some viruses that simulates cells through TLR3 and upregulates the production of IFN-α/β. Before and at 2, 3, 4, 6, 24, and 48 hours after injection of poly I:C, a fixed volume of blood was collected from the tail vein. Erythrocytes were lysed using hypotonic saline, and lymphocytes were counted using a hemocytometer. The cells were stained with CD8α, CD4, and B220 antibodies (Table 3). The number of lymphocytes was analysed with flow cytometry. To calculate the number of circulatory lymphocyte subsets, the absolute number of total lymphocytes was multiplied by the percentage of each lymphocyte subpopulation.

#### 4.1.1.5 FITC skin painting (II)

To evaluate the migration of endogenous DCs, FITC (1%, Thermo Scientific) was dissolved in 1:1 acetone/dibutylphtalae (Sigma) and applied on the dorsal side of the ears of KO and WT mice. 20 and 48 hours later, mice were sacrificed and their auricular dLNs were collected and a single cell suspension was prepared. The samples were stained with CD45, MHCII, CD11c, CD11b or CD11b and CD103 antibodies listed in Table 3. The cells were also permeabilized with 0.5% saponin buffer and stained with a Langerin antibody (Table 3). The number of DCs were obtained with flow cytometry. The DCs were gated by their $CD11c^{+}MHCII^{hi}$ expression and the following populations were defined among them: (1) Langerhans cells, identified by their $Langerin^{+}CD103^{-}$ expression, (2) Dermal DCs (dDCs), identified by their $Langerin^{+}CD103^{+}$ expression, (3) $CD11b^{+}$ dDCs, identified by their $Langerin^{-}CD11b^{+}$ expression, and (4) Double negative DCs (DDCs), identified by their $Langerin^{-}CD11b^{-}$ expression.

#### 4.1.1.6 Exogenous DC migration (II)

For evaluating the migration of exogenous DCs, $1\times 10^{5}$ LPS-matured $DsRed^{+}$ BMDCs in 25µl of PBS were injected s.c into the footpad of KO and WT mice. Twenty hours later, mice were sacrificed and their popliteal dLNs were collected. A single-cell suspension was prepared and stained with CD45, MHCII and CD11c antibodies listed in the Table 3. The number of DCs were acquired with flow cytometry. DCs were identified by their expression of DsRed, CD45, CD11c and a high expression of MHCII.

To visualize DC migration into the lymphatic vessels, LPS-matured BMDCs were stained with CFSE (ThermoFisher) according to the manufacturer's instruction.

$2.5\times10^5$ CFSE-labelled cells were intradermally injected into the ear dermis of WT and KO mice, followed by applying 2% OXA in acetone/olive oil (4:1 volume/volume) on both sides of the ears. Mice were sacrificed one day later, and their ears were collected. The dorsal and ventral sides of the ears were separated, fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton and stained with anti-podoplanin and anti-LYVE-1 antibodies listed in Table 4. The number of transmigrated DCs were examined with fluorescence imaging.

#### 4.1.1.7 Chimeric Mice (II)

To generate BM-chimera mice, Clever-1 KO and WT mice were lethally irradiated two times with 5Gy (Faxitron Multirad 350) with 3-hour intervals. Thereafter, the BM cells of either KikGR photoconvertible transgenic or CD11c-YFP reporter mice were collected and a single-cell suspension was prepared. 10 million cells were injected i.v. into the irradiated recipient mice. Mice (i.e. so-called chimera mice) were let to recover for at least two months.

To track the photoconverted cells and evaluate DC migration via the lymphatic vessels, the shaved belly of Kikume chimera mice (mice reconstituted with BM cells of KikGR photoconvertible transgenic mice) was irradiated with UV light and 2% OXA in acetone/olive oil (4:1 volume/volume) was applied topically. One day later, mice were sacrificed, their axillary dLNs were collected, single-cell suspensions were prepared and stained with CD45, MHCII and CD11c antibodies listed in Table 4. The number of photoconverted DCs were examined with flow cytometry.

To examine the transmigration of the CD11c-YFP DCs into lymphatics, 2% OXA in acetone/olive oil (4:1 volume/volume) was applied on both sides of the ears of CD11c-YFP chimera mice (mice reconstituted with BM cells from CD11c-YFP reporter mice). Twenty hours later, mice were sacrificed, and ears were collected, splitted as described below and stained with LYVE-1 antibody. The number of transmigrated CD11c-YFP DC were analysed from the ventral side of ears using fluorescence imaging. In another set of experiments, the CD11c-YFP chimera mice were used to study the CHS response.

#### 4.1.1.8 DQ-Ovalbumin (DQ-OVA) Injection (II)

For evaluating the functionality of lymphatic vessels, 5 mg/ml of DQ-OVA (Thermo Fisher Scientific) was subcutaneously injected intradermally into the hind footpad of WT and KO mice. The popliteal dLNs were collected 90 minutes later and embedded in OCT, followed by snap freezing on dry ice. The frozen sections were cut and examined by fluorescence imaging. The presence of digested DQ-OVA was analysed by quantifying the emitted green light.

#### 4.1.1.9 Microlymphography (II)

To evaluate the functionality of the lymphatic vessels, tetramethylrhodamine (TRITC)–dextran (molecular weight 2 million Da; Sigma) was injected intradermally into the ear tip of KO and WT mice. The distribution of TRITC-dextran in the ear dermis was immediately examined with a fluorescence microscope (Leica).

### 4.1.2 *Ex vivo* experiments

#### 4.1.2.1 Ex vivo adhesion assay (I)

To evaluate lymphocyte binding to the mouse spleen, frozen spleen tissues of KO and WT mice were cut into 10 µm-thick slices and incubated with $1.5\times10^6$ mouse lymphocytes, isolated from the mesenteric lymph nodes for 30 minutes on rotator at 60 rpm/min, as previously described. (Jalkanen & Butcher, 1985; Salmi et al., 1997) The sections were washed with PBS and fixed with PBS containing 1% glutaraldehyde. Mounted sections were imaged using a dark field microscope. The adhered cells appeared as round cells bound to the splenic vessels. The location of the adhered cells within the different splenic compartments was verified by changing the focus. The number of cells adhered to the different splenic compartments was counted and normalized to the number of cells in the MZ by defining the number of cells in the MZ as 1.0.

To evaluate the binding of lymphocytes to human spleen, frozen human spleens (n=3) were cut into 10 µm-thick slices. Lymphocytes were isolated from blood samples of healthy volunteers by using Ficoll-paque centrifugation and incubated with a mouse monoclonal antibody against Clever-1 (clone 3-372) (Irjala, Johansson, et al., 2003) or with its negative class-matched control antibody AK-1 (InVivo Biotech) for 30 minutes. Thereafter, the spleen sections were incubated with $2\times10^6$ lymphocytes for 30 minutes on a rotator at 60 rpm/min. The samples were then washed and fixed, as described above for the mouse samples. The number of vessels with the adhered cells and the number of cells per vessel were quantified. To allow comparison, the number of attached cells in the control antibody group was considered as 100% attachment. Four sections with approximately 1.5 $cm^2$ of the area were imaged and quantified for each donor's spleen.

#### 4.1.2.2 Generating Bone marrow-derived DCs (BMDCs) (II)

BMDCs were generated from murine BM cells. Briefly, BM cells were cultured with 30 ng/ml of recombinant murine GM-CSF (PEPROTECH) for six days on a bacterial

culture plate. To activate immature DCs, cells were cultured with 500 ng of LPS overnight, as described. (Lutz et al., 1999)

#### 4.1.2.3 Split-Ear Assay (II)

For evaluating the adhesion of DCs to lymphatic vessels, DsRed$^{+}$, CD11c-YFP or WT LPS-matured BMDCs were generated as described previously. BMDCs were then purified using a pan-dendritic cell isolation kit (Miltenyi). The isolated BMDCs were incubated with the splited ventral side of the ears for 20 minutes at 37°C. Samples were washed to remove the nonadherent cells and then incubated for 20 minutes or 2 hours at 37°C in 5% CO2. Ear sheets were washed, fixed with 4% paraformaldehyde and stained with anti-LYVE-1 and anti-podoplanin antibodies listed in Table 4. Samples were then mounted using ProLong™ Gold Antifade Mountant (ThermoFisher). The number of adhered cells in the entire samples were examined with confocal microscopy.

#### 4.1.2.4 Multiplex Analyses

Skin ear and the auricular dLNs of mice at steady-state, one day after OXA sensitization, and one and two days after OXA challenge were collected, lysed, and their proteins were extracted using the ReadyPrepTM Protein Extraction Kit (Bio-Rad). The extracted protein was stored at -70°C until the initiation of the experiment. The protein concentration was measured using DC Protein Assay (Bio-Rad). Total protein (12.5 µg/sample) was used for the multiplex assay, Bio-Plex Mouse cytokines 23-plex assay (Bio-Rad), according to the manufacturer's instructions. Samples were analysed using Bio-Plex 200 system (Bio-Rad).

## 4.2 *In vitro* experiments

### 4.2.1 Purification of Placental Clever-1 (I)

To purify Clever-1 from human placenta, 100 g of placenta was lysed by incubation in lysis buffer (20% Triton X-100, 0.1 M Tris-Base, 1.5 mM MgCl2, and 1.5 M NaCl) overnight at +4°C on a shaker at 180 rpm/min. The crude lysate was then centrifuged and prepurified using Sepharose CL-4B beads (GE Healthcare). Thereafter, the anti-Clever-1 antibody 3-372 (InVivo Biotech) coupled with CnBr-activated Sepharose beads (GE Healthcare) (3 mg/ml beads) was used to affinity-purify the Clever-1 protein. Afterwards, the presipitate was rewashed with lysis buffer, eluted with 50 mM triethylamine and lyophilized. The samples were then rehydrated with Laemmli sample buffer (nonreducing) and separated on a 5 to 12.5%

SDS–polyacrylamide gel electrophoresis (SDS-PAGE) gel (Bio-Rad). The gels were then stained with GelCode Blue Safe Protein Stain (Thermo Scientific). Alternatively, gels were subjected to Western blot analysis with 2 mg/ml of anti-Clever-1 antibody 9-11 (InVivo Biotech) as the primary antibody and IRDye 800CW goat anti-rat (LI-COR Biosciences; 1:20,000) as the secondary antibody.

### 4.2.2 Binding of Purified placental Clever-1 to lymphocytes (I)

To evaluate whether lymphocytes can bind to Clever-1 protein, lymphocytes were isolated from the spleen of Balb/C or C57BL/6N mice. $5\times10^5$ isolated lymphocytes were then incubated with 50 µg of pooled ($n$=2) purified Clever-1 placental protein or bovine serum albumin (BSA) in 1 ml of PBS for 30 minutes at 37°C. To detect the binding of placental protein to lymphocytes, samples were washed and stained with anti-Clever-1 antibody 9-11-AF647 (1 µg/ml) (InVivo Biotech). To quantify the different subsets of lymphocytes, samples were stained with CD45, CD3, CD4, CD8, and CD19 antibodies listed in Table 3. The anti-Clever-1 antibody (InVivo Biotech) recognizes a different non-overlapping epitope than the anti-Clever-1 antibody 3-372 (InVivo Biotech), which was used to purify the placental Clever-1. The binding of placental Clever-1 to lymphocytes was examined with flow cytometry.

### 4.2.3 RNA-extraction and RNA-sequencing (I & II)

RNA was extracted using the RNeasy Plus Micro kit (QIAGEN) or the NucleoSpin RNA kit (Macherey-Nagel). The quality and quantity of RNA samples were verified using Bioanalyzer 2100 (Agilent). The RNA integrity number of the RNA samples were eight or more. The complementary DNA (cDNA) of the single-stranded RNA samples were synthesized using the SMART-Seq v4 Ultra Low Input RNA Kit (Takara). Illumina sequencing libraries were generated using a Nextera XT (Illumina), and the libraries were sequenced with HiSeq 3000 (Illumina). Single-end sequencing chemistry was performed with a reading length of 50 bp at the Finnish Functional Genomics Centre, University of Turku and Åbo Akademi and Biocenter, Finland.

### 4.2.4 Quantitative PCR (I)

Total endothelial RNA was extracted. cDNA was synthesized using SuperScript VILO cDNA Synthesis kit (ThermoFisher Scientific) or iScript cDNA Synthesis Kit (Bio-Rad). Quantitative PCR was performed using TaqMan Gene Expression Assays

(Applied Biosystems) for *IRAK4* and *IFI202b*. The PCR reaction was carried out using the 7900HT Fast Real-Time PCR System (Applied Biosystems/ ThermoFisher Scientific) in the Finnish Microarray and Sequencing Center. In the second publication of this thesis, the Universal ProbeLibrary (UPL) system with probe number 74 and primers was used to evaluate Clever-1 expression.

### 4.2.5 siRNA Silencing of Clever-1 on Human Dermal Lymphatic Endothelial Cells (HDLECs) (II)

To silence Clever-1 expression on HDLECs (Promocell), cells were cultured in MV2 medium (C22022, Promocell) on a fibronectin-coated 12-well plate. One day later, Clever-1 expression was silenced using lipofection with Lipofectamine RNAiMAX (ThermoFisher Scientific) and 15 nM siRNA (ON-TARGETplus siRNA, human STAB1, J-014103-08-0020, Dharmacon). Control cells were treated with a control construct (ON-TARGETplus Non-targeting Control Pool, D-001810-10-20, Dharmacon).

### 4.2.6 Generation of Human Monocyte-Derived DCs (moDCs) (II)

Peripheral blood mononuclear cells were first isolated from buffy coats, purchased from the Finnish Red Cross Blood Service (permit number: 22/2018), using gradient centrifugation (Ficoll-Paque PLUS, GE Healthcare). Monocytes were then extracted using CD14 MicroBeads (Miltenyi Biotec) and cultured with 500 U/ml GM-CSF and 350 U/ml IL-4 (both from Peprotech) for six days. To keep the medium fresh, half of the culture medium was refreshed after three days.

### 4.2.7 Co-culture of Human moDCs and HDLECs (II)

Human moDCs and HDLECs were prepared as described. $5\times10^5$ moDCs were co-cultured with either Clever-1-silenced or control-treated HDLECs in 12-well plates for two days in MV2 medium. Thereafter, samples were washed and stained with antibodies against CD40, CD83 and MHC class II antibodies (Table 3). The cells were analysed with flow cytometry.

### 4.2.8 Mixed Leukocyte Reaction Assay (II)

T-cells were first isolated from the blood of healthy volunteers, using a T-cell isolation kit (EasySep™), and then labelled with 1µM of Vybrant CFDA SE Cell Tracer (CFSE; Thermo Fisher Scientific), according to manufacturer's instructions.

$1\times10^5$ CFSE-labelled T-cells were then co-cultured with $1\times10^4$ human moDCs in the presence of either Clever-1-silenced or control-treated HDLECs in 96-well plates for seven days in a mix of 50% MV2 and 50% complete RPMI medium. Thereafter, samples were stained with CD8 and CD4 antibodies (Table 3) and analysed using flow cytometry.

## 4.3 Flow Cytometry

### 4.3.1 Preparation of single-cell suspensions (I & II)

Single-cell suspensions were prepared from secondary lymphoid organs (I & II), skin (I) and BM (II). For analysing the lymphocytes in the spleen or LN, tissues were mechanically disrupted with a cell strainer, washed with PBS and filtered. When analysing the spleen, erythrocytes were lysed with hypotonic solutions or lysing buffer (BD Bioscience). To prepare single splenic stromal cells (I), samples were first mechanically disrupted and then incubated with an enzymatic cocktail, containing collagenase P (200 µg/ml) (Sigma), dispase (800 µg/ml) (ThermoFisher), and DNase I (100µg/ml) (Sigma) for 30 minutes at 37℃. After complete digestion, the single-cell suspension was then washed with PBS and filtered. For analysing the DCs in the LNs (II), samples were first mechanically disrupted and then enzymatically digested in RPMI containing Collagenase P (500 µg/ml) (Sigma) and DNase I (100µg/ml) for 30 minutes at 37 ℃, followed by adding EDTA in the last 5 minutes of incubation. The single-cell suspension was then washed with PBS and filtered. To isolate the BM cells (II), femurs were collected and the BM cells were flushed out with a needle.

### 4.3.2 Staining and data collection (I & II)

For analysing the obtained single-cell suspensions with flow cytometry, mouse samples were first incubated with purified rat anti-mouse CD16/32 (Mouse BD FC Block) on ice to block the Fc receptors and decrease non-specific binding of antibodies. Thereafter, samples were stained with fluorochrome-labelled antibodies (Table 3). Cells were washed and analysed with the LSR II (I) or LSR Fortessa (I & II) flow cytometers, both from BD Biosciences. The data was analysed using FlowJo software (FlowJo LLC).

**Table 3. Antibodies used in flow cytometry and cell sorting.**

| Antibody | Company | Cat# | Publication |
|---|---|---|---|
| **CD4- Alexa Fluor 647** | BD Bioscience | 557681 | I |
| **CD8α-PerCP-Cy™5.5** | BD Bioscience | 551162 | I |
| **CD45R/B220-Pacific Blue™** | BD Bioscience | 558108 | I |
| **CD45-V450** | BD Bioscience | 560368 | I |
| **CD8-BV650** | BD Bioscience | 565289 | I |
| **CD3-PerCP-Cy5.5** | BD Bioscience | 560835 | I |
| **CD19- BV510** | BD Bioscience | 562947 | I |
| **CD31-APC** | BioLegend | 102509 | I & II |
| **CD45-PerCP-Cy5.5** | BD Biosciences | 550994 | I |
| **CD45-BV650** | BD Bioscience | 563410 | II |
| **CD103-PE** | BD Bioscience | 557495 | II |
| **CD11c-PerCP-Cy5.5** | BD Bioscience | 560584 | II |
| **CD11c-BV421** | BD Bioscience | 562782 | II |
| **CD11b-eFluor450** | BD Bioscience | 48-0112-82 | II |
| **CD11b-APC-Cy7** | BD Bioscience | 557657 | II |
| **MHC II-PE-Cy7** | BD Bioscience | 107629 | II |
| **MHC II-PerCP-eFluor 710** | Invitrogen | 46-0463-82 | II |
| **CD11c-FITC** | BD Bioscience | 553801 | II |
| **CD11c-Alexa Fluor 488** | eBioscience | 53-0114-82 | II |
| **MHCII-PE-Cy7** | Invitrogen | 25-5321-82 | II |
| **CD45-PE** | BD Bioscience | 553081 | II |
| **CD45-APC-Cy7** | BD Bioscience | 557869 | II |
| **CD3- Alexa Fluor 647** | BD Bioscience | 557869 | II |
| **B220-BV421** | BD Bioscience | 562922 | II |
| **CD4-APC-Cy7** | BD Bioscience | 561830 | II |
| **Foxp3-PE-CF594** | BD Bioscience | 562466 | II |
| **CD40-BV510** | BD Bioscience | 563456 | II |
| **CD83-BV421** | BD Bioscience | 562630 | II |
| **CD8-APC** | BD Bioscience | 561952 | II |
| **CD4-PE** | BD Bioscience | 555347 | II |
| **Langerin-AF647** | Dendritics | 929F3.01 | I & II |
| **Anti-Clever-1 (9-11-AF647** | InVivo Biotech | - | I & II |
| **Anti-Clever-1 (372-AF647)** | InVivo Biotech | - | I |
| **CD45-eFluor 700** | BD Bioscience | 560566 | II |
| **CD3-FITC** | BD Bioscience | 555274 | II |
| **CD103-Bv510** | BD Bioscience | 563087 | II |
| **CD25-APC** | BD Bioscience | 557192 | II |
| **CD11b-PE** | BD Bioscience | 553311 | II |
| **CD40-BV510** | BD Bioscience | 563456 | II |
| **CD83- BV421** | BD Bioscience | 562630 | II |
| **MHCII-MHC II** | BD Bioscience | 555558 | II |
| **Podoplanin-APC** | BioLegend | 127410 | II |
| **CD31- Alexa Fluor 488** | BioLegend | 102414 | II |

### 4.3.3 Cell Sorting (I & II)

In the first article, to isolate splenic stromal cells, first, single-cell suspensions (n=3 for each genotype) were prepared. Thereafter, leukocytes ($CD45^+$ cells) were depleted using CD45 microbeads (Miltenyi); the enriched cells were stained with CD31 and CD45 (Table 3) as well as LIVE/DEAD fixable near-IR dead cell stain (Thermo Fisher Scientific). The samples were then washed and sorted using a FACSAria II (BD) equipped with a 100-µm tip (BD). The $CD45^-CD31^+$ cells were directly collected into TRIsure (Bioline) for RNA extraction.

In the second article, the popliteal and brachial LNs were collected from KO and WT mice (n=8 for each genotype) at steady-state and one day after s.c injection of 1mg/ml of OVA (EndoGrade) emulsified in incomplete Freund's adjuvant (Sigma) (1:1). Single-cell suspensions were prepared and leukocytes ($CD45^+$ cells) were depleted using CD45 microbeads (Miltenyi). The enriched cells were stained with CD31 and podoplanin (Table 3) as well as LIVE/DEAD fixable near-IR dead cell stain (Thermo Fisher Scientific). The samples were then washed and sorted using a Sony Cell Sorter equipped with a 100-µm tip. The $CD45^-CD31^+podoplanin^+$ cells were directly collected into TRIsure (Bioline) for RNA extraction.

## 4.4 Microscopic imaging and data analysis

### 4.4.1 Immunohistochemistry (I & II)

In this thesis work, immunohistochemistry was mostly performed on OCT-embedded frozen sections, unless otherwise mentioned. Frozen tissues were cut into the desired thickness and fixed with acetone unless otherwise noted. Samples were either stained with directly conjugated antibodies or a combination of primary and proper secondary antibodies. The antibodies that were used for fluorescence staining are listed in Table 4. Samples were then mounted with Prolong Gold containing DAPI. Alternately, samples were stained with Hoechst (Thermo Fisher Scientific, Cat. 62249) and then mounted with Prolong Gold without DAPI.

**Table 4. Antibodies used in microscopy.**

| Antibody | Company | Cat# | Publication |
|---|---|---|---|
| **MAdCAM-1 (MECA-376)** | E. Butcher | - | I |
| **PLVAP-1 (MECA-32)** | E. Butcher | - | I |
| **CD31** | BD Biosciences | 550274 | I |
| **ICAM-1** | BD Biosciences | 550287 | I |
| **VCAM-1** | BD Biosciences | 550547 | I |
| **E-selectin** | BD Biosciences | 09521D | I |
| **P-selectin** | BD Biosciences | 09481D | I |
| **CXCL13** | R&D Systems | AF470 | I |
| **Goat anti-Rat IgG- Alexa Fluor 488** | Invitrogen | A11006 | I |
| **Goat anti-Hamster IgG- Alexa Fluor 546** | Invitrogen | A21111 | I |
| **Goat anti-Rat IgG- Alexa Fluor546** | Invitrogen | A11081 | I |
| **Donkey Anti-Goat IgG-NorthernLights-NL557** | R&D Systems | NL001 | I |
| **Donkey Anti-Rat IgG- Alexa Fluor 488** | Abcam | ab50153 | I |
| **Biotin anti-mouse CD45.1** | BioLegend | 110704 | I |
| **Clever-1 (9-11)** | InVivo Biotech | - | I & II |
| **Clever-1 (9-11)- Alexa Fluor 647** | InVivo Biotech | - | I & II |
| **Streptavidin- Alexa Fluor 488** | Invitrogen | S11223 | I |
| **CD31-APC** | BioLegend | 102509 | I & II |
| **Podoplanin-PE-Cy7** | BioLegend | 127412 | II |
| **CD3-AF647** | BD Biosciences | 557869 | |
| **B220** | eBioscience | 14-0452-82 | II |
| **LYVE-1** | eBioscience | 14-0443-82 | II |
| **MHC II** | eBioscience | 14-5321-82 | II |
| **anti-mouse IgG1-AF488** | Life Technologies | A21121 | I |
| **Anti-Clever-1 (372)** | InVivo Biotech | - | I |
| **Podoplanin** | BioLegend | 127410 | II |

### 4.4.2 Image acquisition (I & II)

Non-fluorescent stained tissues were visualized using a Leica dark-field microscope. Fluorescent images were acquired using LSM 780 and LSM 880 confocal microscopes, equipped with ZEN imaging software (both from Carl Zeiss SAS, Germany). Images were acquired with Plan-Apochromat 20x/0.8, C-Apochromat Korr M27 40x/1.20, and C-Apochromat 63× /1.2 oil objectives. Images were also acquired with a 3i spinning disk microscope [Intelligent Imaging Innovations (3i)], equipped with SlideBook 6 software and Plan-Apochromat 10× /0.45, 20× /0.8, 63× /1.4, and LD c-apochromat 40× /1.1 W objectives. Raw images were analysed using the ImageJ software (Rasband, W.S., ImageJ, U.S. National Institutes of Health, Bethesda, MD, USA) and SlideBook 6 and Imaris 8 (Bitplane Inc).

### 4.4.3 Bioinformatics (I & II)

To analyse the RNA sequencing data acquired in the first article, STAR version 2.5.2b was used to align the single-end 50-base pair reads to the mm10 mouse genome. Reads were counted using the Subreads package and then normalized using the trimmed mean of M-values normalization method of the edge R R/Bioconductor package. The difference in gene expression between the samples was identified using a Benjamini-Hochberg adjusted P value [false discovery rate (FDR)] <0.05 and 2-fold changes ((log2ratio ≥1 and ≤-1). The functional enrichment analysis of differentially expressed genes (DEG) was then performed by gene ontology (GO) database using topGO and GOstats and their associated annotation packages.

In the second article, FASTQ data was analysed using the BaseSpace Sequence Hub (Illumina). Briefly, the quality of the data was first checked using the FastQC application in BaseSpace. Thereafter, sequences were aligned against the mouse reference genome (mm10) (UCSC, RefSeq gene annotation) using the RNA-Seq Alignment application. The STAR aligner (version 2.5.2b) was used for read mapping, and Salmon was used to quantify the reference genes and transcripts. The RNA-Seq Differential Expression application using DESeq2 was used to identify gene expression differences between the samples. The genes exhibiting at least 2-fold changes ((log2ratio ≥1 and ≤-1) and FDR<0.05 were considered significant and as DEG. All RNA sequencing data is uploaded into Gene Expression Omnibus (GEO) under the GEO accession number GSE148730. The DEGs were further analysed using QIAGEN Ingenuity Pathway Analysis (QIAGEN IPA). The Venn diagrams were generated with the Venn diagram tool at http://bioinformatics.psb.ugent.be/webtools/Venn.

### 4.4.4 Statistical Analyses (I & II)

The sample size was based on previously published literature and pilot experiments. None of the experiments in this thesis work was conducted in a blinded fashion, and no specific randomization method was used. The numerical data is given as mean ± SEM. The different groups were compared by calculating the student's t-test with Welch's correction (when the variances were significantly different) or the Mann-Whitney U test. Multiple comparisons were done using one-way analysis of variance (ANOVA) with Tukey's test. Statistical data were analysed using GraphPad Prism software v8. For analysing the RNA-seq data, R-package were used. P values under 0.05 were considered statistically significant (*P < 0.05, **P < 0.01, and ***P < 0.001). The outliers were identified and removed using the Grubbs' test.

# 5 Results

## 5.1 Clever-1 regulates lymphocytes trafficking into lymphoid organs (I)

Clever-1 has been shown to be expressed on efferent and afferent lymphatic vessels where it regulates lymphocyte migration within lymphatics. (Irjala, Alanen, et al., 2003; Karikoski et al., 2009) However, its role in lymphocyte trafficking into secondary lymphoid organs is not completely understood. Therefore, we aimed to study whether Clever-1 regulates lymphocyte migration into the dLNs via the HEVs. To discriminate between lymphocyte entry via the HEVs and lymphocyte egress from the efferent lymphatics, we injected poly (I: C) prior to intravenous injection of CFSE labelled lymphocytes. Poly(I: C) upregulates IFN-α/β and rapidly induces transmembrane C-type lectin CD69 on lymphocytes and inhibits $S1P_1$, resulting in lymphocyte retention in the lymphoid organs. (Shiow et al., 2006) Analysing the blood of KO and WT mice at different time points showed that Clever-1 KO mice treated with poly(I:C) significantly retained a greater number of lymphocytes in their blood circulation at the early time points (2 to 3 hours), compared to their WT controls (I: Figure 1A). However, this difference disappeared after 6 hours, which might be due to the rapid circulation of leukocytes in mice (I: Figure 1A). Further analysis of the blood lymphocyte subpopulations showed that all subpopulations, including B-cells, $CD4^+$ and $CD8^+$ T-cells, were increased (I: Figure 1, B-D). These results suggested that the absence of Clever-1 may impair the migration of lymphocytes into secondary lymphoid organs. Therefore, we next aimed to investigate lymphocyte migration into spleen. Lymphocytes were labelled with CFSE cell tracker and intravenously injected into Clever-1 KO and WT mice. The number of $CFSE^+$ transferred lymphocytes in the spleen, LNs and blood was analysed 2 hours later with flow cytometry. Migration of lymphocytes into the spleen of KO mice was reduced by approximately 50% when compared to the spleen of their WT controls. Further analysis of the lymphocyte composition showed that the number of $B220^+$ B-cells and $CD8^+$ T-cells migrating to spleen was significantly reduced, while the number of $CD4^+$ T-cell remained comparable in the absence of Clever-1 (I: Figure 1E). We also analysed the migration of transferred lymphocytes into mesenteric and peripheral LNs and did not find any significant difference in

lymphocyte migration between WT and KO mice (I: Figure 1F, S1A-B). Despite the impaired lymphocyte migration into the spleen, the number of transferred lymphocytes in the blood also remained comparable (I: Figure S1A, C).

## 5.2 A new route for lymphocytes to enter the spleen (I)

To further investigate the possible role of Clever-1 in lymphocyte migration into the spleen, we first analysed the expression of Clever-1 on splenic vasculature. We found that Clever-1 is exclusively expressed on the blood vessels of RP but absent from the vessels of MZ and WP (I: Figure 2A-B). The selective expression of Clever-1 on RP blood vessels together with the observation of reduced lymphocyte entry into the spleen in the absence of Clever-1 led us to hypothesise that lymphocytes mainly enter the spleen via the RP blood vessels. To explore the localisation where lymphocytes attach to the vascular endothelium and whether their attachment is Clever-1 dependent, we performed mouse *ex vivo* adhesion assays, in which lymphocytes were incubated on spleen sections for a short time, followed by quantification of the adhered cells. Our results showed that most lymphocytes attached to the RP blood vessels compared to other regions of the spleen. Indeed, the number of adhered lymphocytes to the RP blood vessels was 2.8 times more than that in the MZ, while only a few cells were bound to the WP vasculature (I: Figure 4A-B). Further analysis of adhered lymphocytes showed that the lack of Clever-1 only affects the adherence of the cells to the RP vasculature while it has no significant effect on the binding of the cells to other regions of the spleen, including the MZ and WP (I: Figure 4B-C).

In addition to mice, we also analysed the expression of Clever-1 and its role in human splenic vasculature. Similar to mice, the human RP blood vessels also expressed high levels of Clever-1 (I: Figure 4D). We also performed a human *ex vivo* adhesion assay, in which spleen sections were treated with anti-Clever-1 antibody prior to incubating with lymphocytes. The anatomical structure of the human spleen is similar to mice, but unlike mice, the human spleen does not have the MZ. Therefore, to differentiate the RP vasculature from the WP vasculature, we used CD31, which is highly expressed on the WP but faintly and more diffusely on the RP blood vessels. Quantification of adhered cells showed that anti-Clever-1 treatment significantly decreased the number of adhered cells by 65% in the RP vasculature. The number of vessels with adhered cells was also reduced but not significantly (I: Figure 4E). These results support the mouse data in which Clever-1 supports lymphocyte adhesion to the RP vasculature and their homing into the spleen.

To further validate whether lymphocytes enter the spleen via the RP blood vessels, we performed an adoptive transfer experiment, in which $CD45.1^+$ lymphocytes were transferred into $CD45.2^+$ recipient mice. The number and localization of the transferred cells in the spleen were analysed with confocal microscopy. In line with our previous results, the majority of transferred $CD45.1^+$ cells were found in the RP after 10 minutes, whereas significantly fewer cells were in the MZ, and only a few cells were found in the WP. In contrast, at later time points, more cells appeared in the MZ and WP (I: Figure 5A-D). These data suggest that most of the lymphocytes first enter the spleen via the RP blood vessels and then disperse into other regions of the spleen.

We further confirmed the role of Clever-1 in lymphocyte entry into the spleen via the RP vessels at fixed early time points. In this set of experiments, $CFSE^-$labelled lymphocytes were adoptively transferred to KO and WT mice. The number and localization of the transferred cells in different regions of the spleen were analysed after 10 minutes. Consistent with our previous results, most cells were found in the RP in a Clever-1 dependent manner (I: Figure 5E). Interestingly, the number of transferred lymphocytes in the MZ was comparable between WT and KO mice, whereas the number of lymphocytes in RP was significantly less in KO mice, suggesting a significant role of Clever-1 in lymphocyte trafficking into the spleen via the RP blood vessels (I: Figure 5E).

As CFSE has been reported to impair lymphocyte migration (Nolte, Kraal, et al., 2004), we used $DsRed^+$ reporter mice to further validate these results at later time points. Flow cytometry and immunofluorescence analyses of the recipient spleens after 2 hours showed that homing of transferred $DsRed^+$ lymphocytes is significantly reduced in the absence of Clever-1 (I: Figure 5F-G). Further immunohistochemistry analyses of the spleen samples showed that the number of transferred lymphocytes in the splenic RP and MZ was significantly less in KO mice than in their WT controls. The number of lymphocytes in the WP remained comparable between KO and WT mice (I: Figure 5H).

As Clever-1 has been reported to be upregulated on blood vessels at sites of inflammation, we mimicked Gram-negative bacterial infection in mice by intraperitoneal injection of LPS to investigate whether inflammation can affect Clever-1 expression on the splenic vasculature and subsequently leukocyte migration into the spleen. The expression level of Clever-1 on $CD31^+$ BECs was analysed 18 hours later. Clever-1 expression, as well as lymphocyte homing into the spleen, was reduced 18 hours after i.p. administration of LPS (I: Figure S2B-C). In contrast to lymphocytes, the number of $CD11b^+/Gr\text{-}1^+$ neutrophils in the spleen remained comparable between the KO and WT mice (I: Figure S2D). However, it appeared that the downregulation of Clever-1 on WT BECs by LPS slightly but significantly increased the migration of $CD11b^+/Gr\text{-}1^+$ cells into the spleen in LPS-

treated WT mice compared to PBS-treated WT mice, whereas no increase was observed between LPS- and PBS-treated KO mice, suggesting a possible role of Clever-1 in the trafficking of myeloid cells upon inflammation (I: Figure S2D). Although Clever-1 is expressed on a subset of macrophages (Kzhyshkowska, 2010), our image analyses showed that it is not expressed by splenic macrophages (I: Figure S2A).

## 5.3 Normal structure of the spleen in the absence of Clever-1 (I)

To investigate whether genetic ablation of Clever-1 affects the anatomical structure of the spleen, we analysed the area of the different splenic compartments, including RP, WP, MZ, as well as the T-cell and B-cell zones. We did not find any statistically significant difference in the area of splenic compartments between KO mice and their WT controls (I: Figure 3A-C). Moreover, the T-cell and B-cell zones were comparable in KO and WT mice (I: Figure 3D-E). These data together showed that Clever-1 deficiency does not affect the anatomical structure of the spleen.

To further validate whether deletion of Clever-1 affects the expression of other adhesion molecules, we performed RNA-sequencing of the isolated splenic $CD45^-$ $CD31^+$ BECs. No significant difference was observed in the expression of *Selp* (encoding P-selectin), *Sele* (encoding E-selectin), *MAdCAM-1*, *Icam-1*, *Vcam-1*, plasmalemmal vesicle-associated protein (*Plvap*), and CD31 between KO mice and their WT controls (I: Figure 2C). As the peripheral node addressin antibody (MECA-79) recognises a carbohydrate epitope, which is expressed on several protein backbones, such as MAdCAM-1 (Berg et al., 1993), CD34 (Baumhueter et al., 1993), glycosylation-dependent cell adhesion molecule-1 (GlyCAM-1) (Lasky et al., 1992), we also compared the mRNA levels of *Podxl2* (encoding podocalyxin), Cd300Ig (encoding nepmucin), *Cd34*, *Emcn* (encoding endomucin), and *Glycam1* in KO and WT mice, and no significant differences were observed (I: Figure 2C). We further validated the expression of these molecules at the protein level, and no significant difference in the morphology and expression of these molecules was observed in KO mice compared to their WT controls (I: Figure 2D). Collectively, these data suggest that deletion of Clever-1 does not affect the phenotype of the splenic vasculature or the anatomical structure of the spleen.

## 5.4 Clever-1 directly binds to B-cells and $CD8^+$ T-cells (I)

To investigate whether Clever-1 can directly bind to lymphocytes, we incubated splenocytes with human affinity-purified Clever-1 followed by flow cytometry

staining with anti-Clever-1 antibody (I: Figure 6A-C). Our data showed that human Clever-1 can directly bind to B-cells and to a lesser extent to $CD8^+$ T-cells, suggesting that Clever-1 expressed on endothelial cells can directly support the migration of lymphocytes. However, Clever-1 did not bind to $CD4^+$ T-cells (I: Figure 6A-C). These results are in line with Clever-1 dependent migration of $CD8^+$ T-cells and $B220^+$ B-cells into the spleen.

## 5.5 Downregulation of CXCL13 on splenic vasculature (I)

To investigate the molecular mechanism behind the impaired migration of lymphocytes into the spleen in the absence of Clever-1, we further analysed the genetic differences between splenic $CD45^-CD31^+$ BECs of KO and WT mice (I: Figure S3A). We found that 117 genes were significantly downregulated, and 34 genes were significantly upregulated in isolated KO splenic BECs compared to their WT controls (I: Figure 7A-B). In the gene ontology (GO) analysis, one of the significantly enriched GO terms was "Leukocyte migration" in KO splenic BECs compared to their WT controls ($P$ = 0.0086; I: Figure 7C). All the seven genes associated with this GO term were downregulated in KO splenic BECs compared to their WT controls (I: Figure 7C). Among these seven genes, interleukin-1 receptor-associated kinase 4 (*Irak4*) and *Cxcl13* were the most significantly affected genes in the absence of Clever-1 (I: Figure 7C-D). Furthermore, interferon-inducible gene 202B (*Ifi202b*) was highly expressed on the KO splenic endothelial cells, whereas it was absent from the WT endothelium (I: Figure 7D).

To validate the expression of *Cxcl13* at the protein level, we performed immunohistochemistry staining of spleen sections and quantified the expression levels of CXCL13 on $CD31^+$ vessels. The expression of CXCL13 was significantly downregulated on the KO splenic RP endothelium compared to WT spleen. The expression of CXCL13 on the WP endothelium remained comparable between KO and WT mice (I: Figure 7E, S3B).

To confirm whether the changes of *Irak4* and *Ifi202b* in the absence of Clever-1 occurred at the transcriptional levels, we quantified the *Irak4* and *Ifi202b* mRNA in the KO and WT splenic BECs by quantitative polymerase chain reaction (qPCR). In line with RNA-seq data, *Irak4* was significantly downregulated in KO splenic BECs compared to their WT controls (p=0.029). More importantly, while we did not observe any expression of *Ifi202b* in the splenic BECs of WT, the *Ifi202b* signal was low but at detectable level in KO BECs ($P$=0.008) (I: Figure S3A).

## 5.6 Clever-1 on lymphatics mediates DC migration (II)

Previous studies have demonstrated Clever-1 expression on a subset of endothelial cells, as well as afferent and efferent lymphatic vessels. Moreover, Clever-1 has been shown to mediate the migration of lymphocytes via afferent lymphatics. (Karikoski et al., 2009) However, its expression on peripheral lymphatic vessels and its role in DC exit from the periphery via the peripheral lymphatic vessels has remained largely unknown. In this study, we first investigated the expression of Clever-1 on peripheral lymphatic vessels with fluorescence imaging and found that Clever-1 is continuously expressed on both collecting and capillary lymphatic vessels both at steady-state and during inflammation (II: Figure 1H, S1B, H). Our data also showed that Clever-1 is expressed on lymphatic, medullary and subcapsular sinuses of the LNs (II: Figure S1A). However, Clever-1 is absent in DCs and lymphocytes (IMMGEN; http://rstats.immgen.org/Skyline/skyline. html).

To examine the migration of DCs via the peripheral lymphatics in the absence of Clever-1, we performed FITC painting experiments at different time points. Quantification of $FITC^+$ DCs after 20 hours in the dLNs showed that the migration of Langerhans cells and $CD103^+$ dDCs were significantly impaired in the absence of Clever-1. In contrast, the migration of double negative dDCs remained comparable between KO and WT mice. The results remained comparable after 48 hours of FITC painting (II: Figure 2A). To validate these results in a different setting, we injected $DsRed^+$ BMDCs into the footpad of KO and WT mice and analysed the migration of transferred cells into the popliteal dLNs after 20 hours. Consistently, the migration of $DsRed^+$ DCs into the popliteal dLNs of KO mice was significantly decreased by 46% in KO mice compared to their WT controls (II Figure 2D).

Clever-1 is an immunosuppressive molecule and its expression has been associated with an anti-inflammatory phenotype of leukocytes and immunosuppressive microenvironment. (Viitala et al., 2019) Therefore, lack of Clever-1 in KO mice can induce a proinflammatory microenvironment and indirectly affect DC migration. To avoid this, we generated Kikume chimeric mice to selectively delete Clever-1 expression from non-hematopoietic cells, while the leukocytes remained with a normal expression level of Clever-1. In this set of experiments, we used KikGR photoconvertible transgenic mice (i.e. Kikume mice) as donors to reconstitute the BM of lethally irradiated Clever-1 KO and WT mice and generate Kikume chimeras. After a full BM reconstitution, the shaved belly of Kikume chimeras was irradiated with UV light followed by topical application of 2% OXA (II: Figure 2B). The quantification of photoconverted DCs in the axillary dLNs of KO chimaeras after 18-hours showed that the migration of DCs into the dLNs was impaired in the absence of Clever-1 on lymphatics (II: Figure 2C).

Since an impaired number of DCs in peripheral tissues essentially leads to lower number of DCs in the dLNs, we next quantified the number of leukocytes and DCs and their subpopulations in the LNs and ear skin of KO mice at steady-state with flow cytometry and fluorescence imaging. No significant difference was observed in the total number of leukocytes and DCs in the ear of KO and WT mice (II: Figure S1E-F). Analysing the lymphocyte subpopulations in LNs showed that the numbers of migratory DCs and B-cells were significantly lower in KO mice compared to their WT controls. In contrast, the number of $CD4^+$ T-cells was significantly higher in KO mice, while the number of resident DCs and $CD8^+$ T-cells remained comparable between KO and WT mice (II: Figure 1A-G).

## 5.7 Normal structure of the lymphatic vessels in the absence of Clever-1 (II)

As any abnormality in lymphatic structure or their functionality can disrupt DC migration, we performed a set of experiments to examine the structure and functionality of lymphatic vessels in KO mice. We first examined the anatomy of the lymphatic vessels with whole-mount ear imaging. Quantification of the area of $LYVE\text{-}1^+$ $Podoplanin^+$ lymphatics showed no significant difference in the morphology or the occupied area of lymphatic vessels in KO and WT mice (II: Figure S1C-D).

To examine lymphatic functionality, we first performed microlymphography by injecting TRITC-dextran into the ear dermis and quantified the area and the fluorescent intensity of TRITC in the drained lymphatic vessels. We did not observe any significant differences in either the fluorescent uptake or distribution of flow of TRITC by the cutaneous lymphatics between the KO and WT mice, indicating normal function of lymphatic vessels in KO mice (II: Figure 1 I-K). To further investigate the lymph flux into the dLNs via the peripheral lymphatics, we used DQ-OVA as a tracer molecule. Due to being heavily fluorescently labelled, the unprocessed form of DQ-OVA has an auto-quenching effect and is undetectable. The auto-quenching effect is lost upon proteolytic digestion, i.e., after ingestion by phagocytes, and emits a bright fluorescent light. The quantification of DQ-OVA signal in the popliteal dLN of KO and WT mice 90 minutes later demonstrated no significant difference in the antigen delivery system via the lymphatics between the KO and WT mice (II: Figure 1L). These results clearly show that lymphatic morphology or functionality is not the underlying reason for the observed impaired migration of DCs in KO mice.

## 5.8 Impaired DC transmigration across the lymphatic vessels (II)

To migrate into the LNs from the periphery, DCs need to first attach, then roll, and eventually transmigrate into the lymphatic vessels. To investigate the underlying mechanism of reduced DC migration, we first asked whether DCs can normally adhere to Clever-1 KO peripheral lymphatic vessels. We performed a set of experiments, so-called split ear assays, in which matured BMDCs were incubated on the exposed lymphatic vessels of the ear dermis. Quantification of the adhered BMDCs to the LYVE-1$^+$ lymphatic vessels after 20- and 120-minutes showed a comparable number of adhered BMDCs between KO and WT mice (II: Figure 3A).

We also tested DC adhesion to the OXA-inflamed KO lymphatic vessels. To our surprise, and in contrast to our steady-state results, a greater number of BMDCs were attached to Clever-1 KO lymphatic vessels after 120 minutes (II: Figure 3B). Collectively, these results suggest that DC adhesion to the lymphatics cannot be the underlying reason for the impaired DC migration into the dLN in KO mice.

We next examined the DC transmigration into the lymphatic vessels by injecting FITC$^+$ matured BMDCs into the ear dermis of KO and WT mice and quantified the number of transmigrated DCs inside the lymphatic lumen (i.e., so-called luminal DCs) one day later. The number of luminal DCs in KO mice was reduced by 30% compared to their WT controls (II: Figure 3C-D). These results clearly show that even though DCs can normally adhere to the lymphatic vessels in the absence of Clever-1, they cannot efficiently transmigrate into the lymphatic vessels.

## 5.9 Clever-1 enhances the antigen-specific immune response (II)

Considering the reduced migration of DCs, we then asked whether the antigen-specific immune response in the dLNs is affected by the lack of Clever-1. To study this, we injected OVA-peptide-loaded DCs into the footpad of KO and WT mice, followed by i.v. injection of CFSE labelled CD4$^+$ OT II T-cells. The number of transferred CD4$^+$ T-cells and their proliferation were analysed with flow cytometry after 2 days. Surprisingly, the total number of transferred OT II CD4$^+$ T-cells in the popliteal dLNs of KO mice was significantly higher by 37.9 ± 8%, compared to WT controls. These results clearly show a more profound antigen-specific immune response in the dLNs of KO mice (II: Figure 4A). Further analysis of the proliferation of CFSE$^+$ cells showed that transferred OT II CD4$^+$ T-cells proliferated 87.3 ± 22% more in the popliteal dLN of KO mice than in WT mice (II: Figure 4A).

The interaction between MHC II molecules expressed on DCs and TCRs on T-cells is fundamental for T-cell activation. Therefore, we studied MHC II expression on DCs to investigate the underlying mechanism of the observed enhanced antigen-

dependent immune response in KO mice. The quantification of MHC II expression on DCs one day after injection of OVA emulsified in incomplete Freund's adjuvant showed a significantly higher expression of MHC II on DCs in the dLN of KO mice compared to their WT controls (II: Figure 4B). However, no significant difference was observed in CD40 expression on DCs between KO and WT mice (II: Figure S1G). Immunohistochemical analysis also showed a higher expression of MHC II in the LNs of KO mice compared to their WT controls at homeostasis (II: Figure 4C). These results indicate that DCs in the Clever-1 deficient microenvironment become more activated and therefore are capable in activating more T-cells on a per cell basis than DCs in WT controls.

## 5.10 Clever-1 affects the immunomodulatory function of LECs (II)

Recently, an important role of LECs in modulating adaptive immune responses has emerged. LECs have been shown to present antigens on their MHC molecules, but due to their lack of costimulatory molecules, they induce tolerance. (Card et al., 2014) In this study, we aimed to investigate the role of Clever-1 on LECs in modulating the immune response. As primary mouse LECs do not retain Clever-1 expression *ex vivo*, we used HDLECs and silenced the expression of Clever-1 by siRNA. Clever-1 expression on Clever-1 siRNA-treated (siClever-1) HDLECs was decreased by 74% at the mRNA level and 65% at the protein level compared to control siRNA (siControl) (II: Figure S4A).

To study the effect of Clever-1 on LECs in DC activation and subsequently T-cell proliferation, moDCs from different healthy human donors were cocultured with siClever-1- or siControl-transfected HDLECs for three days. Quantification of activation markers expressed by moDCs showed that the expression of MHC II, CD40 and CD83 were significantly increased when cocultured with siClever-1 treated HDLECs compared to their siControl treated group (II: Figure S4B). We next cocultured CFSE-labelled human T-cells with siClever-1- or siControl-treated HDLECs in the presence of moDCs from different donors for seven days. In line with our mouse results, $CD4^+$ T-cells proliferated to a greater extent when cocultured with Clever-1-silenced HDLECs, compared to their control-treated group (II: Figure S4 C). These results clearly show that Clever-1-deficient LECs are not able to down-modulate DC activation.

To dissect the underlying mechanism, we studied the genetic differences between Clever-1 KO-LECs and their WT-control-LECs. Both the KO and WT LECs expressed high levels of LEC markers, including *Prox-1, Pdpn, Peccam-1* and *Flt4* with a comparable expression between KO and WT control LECs. However, the expression of *Pdpn* was slightly but statistically significantly lower in KO LECs

than in their WT controls (II: Figure S2A). RNA-seq analysis of LN-LECs showed downregulation of 75 genes and upregulation of 87 genes in KO-LECs at steady-state compared to WT-control-LECs (II: Figure 5A). Pathway analysis of these genes with Ingenuity Pathway Analysis (IPA) revealed overexpression of genes associated with immune response regulation, such as inflammatory response and cell proliferation of T lymphocytes (II: Figure 5B). Also, the pathways related to adhesion of immune cells and binding of professional phagocytic cells were upregulated in KO-LECs, whereas a pathway associated with the transmigration of leukocytes were downregulated in KO-LECs compared to WT-LECs (II: Figure 5B). Further investigating the genes involved in these pathways showed that indeed Clever-1 deficient LECs express higher levels of pro-inflammatory genes, including *Il1b*, *S100A9*, *Ifi202b* and *Csf3,* compared to WT-LECs (II: Figure 5C). We also performed IPA upstream regulator analysis to explain the observed pro-inflammatory state in KO mice. We found that while the pro-inflammatory cytokine IL-2 was predicted to be upregulated, the anti-inflammatory cytokine IL-10 was predicted to be downregulated in KO-LECs compared to WT-LECs (II: Figure 5D). This clearly shows that the proinflammatory signalling cascade is dominant in Clever-1-deficient LECs at steady-state. To validate the higher proinflammatory state in Clever-1-deficient microenvironment, we analysed the cytokine levels in protein lysates of whole LNs. In line with our observations, we found that KO mice have significantly lower levels of anti-inflammatory cytokines, including IL-4, IL-10, IL-13 and CCL2, compared to WT controls (II: Figure 5E). These results support our findings that Clever-1-deficient LECs are incapable of down-modulating the immune response leading to higher activation of DCs and T-cells.

We also investigated the effect of inflammation on the characteristics of KO-LECs. RNA-seq analysis of dLNs-LECs was performed one day after s.c footpad injection of OVA emulsified in complete Freund's adjuvant. While WT LECs downregulated 274 genes and upregulated 400 genes upon inflammation, the Clever-1 KO LECs downregulated an additional 899 genes and upregulated 326 genes (II: Figure 5F). IPA pathway analysis revealed that these genes were associated with the downregulation of the adaptive immune response and leukocyte migration/extravasation pathways in the inflamed KO-LECs compared to their steady-state. Interestingly, a pathway related to the "accumulation of dendritic cells" was significantly upregulated in the inflamed KO-LECs (II: Figure 5G). Among the genes associated with these pathways, *Ackr2*, *Csf2*, and *Tlr3* were significantly downregulated in the inflamed KO-LECs. In contrast, the pathways related to inflammatory response were upregulated in the inflamed WT-control-LECs, compared to their steady-state (II: Figure 5G and S2C-D).

## 5.11 Clever-1 dampens the inflammatory response to a hapten antigen (II)

The CHS reaction is a standard animal model to investigate leukocyte mediated host immune response to a specific antigen. In this model, different subsets of leukocytes, including DCs and lymphocytes, in particular T-cells, contribute to mounting an effective immune response. Here, we aimed to investigate the role of Clever-1 in the whole inflammatory cascade in the CHS model. We first tested whether inflammation induced by OXA affects Clever-1 expression on lymphatics. We did not find any significant difference in the expression of Clever-1 on the inflamed ear lymphatic vessels (II: Figure S1H). Measuring the ear thickness of KO and WT mice at different time points after OXA-challenge showed that KO mice failed to develop a robust inflammatory reaction to OXA compared to their WT controls (II: Figure 6A).

We next investigated whether the impaired inflammatory response in KO mice is caused by impaired lymphocyte migration to the inflamed tissues in the elicitation phase, or whether Clever-1-deficiency also affects the phenotype of the lymphocytes. We first analysed the leukocyte compositions in the inflamed KO and WT ears two days after the OXA challenge. We found that the migration of $CD8^+$ T-cells into the OXA-challenged-ears was significantly reduced in the absence of Clever-1, while the number of $CD4^+$ T-cells remained comparable between WT and KO mice (II: Figure 6B-C). Further analysis of the subpopulations of $CD4^+$ T-cells showed that there tended to be more $FOXP3^+CD25^+$ regulatory T-cells in KO ears (II: Figure 6D). $FOXP3^+CD25^+$ $CD4^+$ T-cells have been reported to be the most immunosuppressive T-cells among the $FOXP3^+$ regulatory $CD4^+$ T-cells. (Ikebuchi et al., 2016)

Even though Clever-1 is not expressed on the blood vessels at steady-state, it is rapidly upregulated upon inflammation and is involved in leukocyte migration into inflamed tissues. (Irjala, Johansson, et al., 2003; Salmi et al., 2004) On the other hand, Clever-1 has been shown to regulate lymphocyte egress from the LNs. (Irjala, Johansson, et al., 2003; Salmi et al., 2004) Therefore, we next asked whether the impaired CHS response is caused by defective lymphocyte egress from the dLNs after sensitisation or rather by impaired lymphocyte migration into the inflamed ears via the BECs. We adoptively transferred OXA-primed $DsRed^+$ lymphocytes, isolated from dLNs of OXA-sensitized WT mice, into naïve mice via their tail vein, followed by application of OXA on their ears. Ear swelling was measured at different time points. Similar to our previous results, KO mice failed to initiate a robust inflammatory response against the OXA, compared to their WT controls (II: Figure 6E). However, the difference in the ear swelling between KO and WT mice was not as large as it was in the conventional model. These results suggest that the observed failure in the initiation of the CHS response in KO mice is due to both reduced

lymphocyte egress from the dLN as well as reduced entrance into the inflamed ears via the BECs. Indeed, quantification of the number of the adoptively transferred OXA-primed DsRed$^{+}$ lymphocytes from the whole-mount imaging of the inflamed ears showed that the migration of transferred cells into the inflamed ears was significantly impaired in the absence of Clever-1 (II: Figure 6F-G).

We also analysed cytokine levels in the whole protein lysate of ear skin at steady-state and 24 hours after OXA-sensitization, as well as one and two days after the OXA challenge. Interestingly, while the levels of inflammatory cytokines, including IFN-ɣ, IL-1α, IL-17 and TNFα, were higher at steady-state in the ears of KO mice, they were rapidly downregulated upon inflammation and became comparable to WT mice (II: Figure 6H, S3). More interestingly, the level of the anti-inflammatory cytokine IL-4 was comparable at steady-state, whereas it was significantly upregulated one day after the OXA challenge in the ears of KO mice (II: Figure 6H).

Macrophages have been shown to play an important role in the CHS response. (Natsuaki et al., 2014) As Clever-1 is also expressed on a subset of macrophages (Viitala et al., 2019), we next generated CD11c-YFP chimeras to rule out the possibility that the impaired DC migration and CHS response are due to the contribution of Clever-1-deficient macrophages (II: Figure 7A). Similar to our previous results, ear swelling of CD11c-YFP chimeric mice was also significantly attenuated one day after the OXA challenge (II: Figure 7B). Whole-mount imaging of one-day OXA sensitised ears and their auricular dLNs also showed that, in line with our previous results, the migration of the DCs into the dLNs was impaired in the absence of Clever-1 (II: Figure 7C-G). Moreover, quantification of the number and localization of luminal CD11c-YFP$^{+}$ DCs showed that the transmigration of DCs was impaired in the absence of Clever-1 (II: Figure 7E).

# 6 Discussion

## 6.1 Clever-1 regulates leukocyte migration into lymphoid organs (I, II)

The structure and the role of secondary lymphoid tissues are one of the most compelling narratives in immunology. Lymphoid tissues connect the innate and adaptive immunity in a unique organized way. Lymphocytes constantly recirculate between blood and secondary lymphoid organs searching for antigens. The spleen is the largest secondary lymphoid organ, where leukocytes can encounter blood-borne antigens and initiate adaptive immune responses. Splenic RP blood vessels are generally known to be open-ended, feeding an open microcirculation, and thus, blood can freely enter the spleen parenchyma without passing through a regular endothelial barrier. (Buyssens et al., 1984; Kusumi et al., 2015; Nolte et al., 2002; Schmidt et al., 1988; B. Steiniger et al., 2007; B. S. Steiniger, 2015; B. S. Steiniger et al., 2014)

On the other hand, the known adhesion molecules and their receptors that mediate lymphocyte migration into the LNs have been shown to be dispensable in lymphocyte trafficking into the spleen. (Nolte et al., 2002) For instance, ICAM-1 and MADCAM-1 are involved in lymphocyte migration into the mesenteric LNs via the HEVs, and both are predominantly expressed on the sinus lining cells in the MZ. However, different functional studies have excluded their role in lymphocyte homing into the spleen. (Kraal et al., 1995; Nolte et al., 2002) Taken together, the open-ended vessels and lack of regulatory adhesion molecules implied that lymphocytes passively enter the spleen with the blood flow without passing the endothelial barriers and without involvement of conventional regulatory adhesion molecules and their receptors. (Kusumi et al., 2015; Nolte et al., 2002; Schmidt et al., 1988; B. Steiniger et al., 2007; B. S. Steiniger, 2015)

In this study, we found that Clever-1 is exclusively expressed on the RP blood vessels and its absence reduces lymphocyte migration to the spleen by 50%. In addition, analysing the migratory pattern of lymphocytes into the spleen versus LNs showed that transferred B-cells tend to migrate to the spleen more than LNs, whereas transferred T-cells tend to migrate to the LNs more than the spleen (Figure 1E–F). These data suggest that lymphocyte migration into the spleen is not a passive process

forced by the blood flow, but rather an active process, which is regulated by Clever-1. Indeed an early intravital microscopy study showed that lymphocytes first adhere to the venous endothelium to migrate into the spleen parenchyma, and then slowly roll along the vessel walls, independent from the direction and magnitude of the blood flow, suggesting that lymphocyte migration into the spleen is not a passive process forced by the blood flow. (Schmidt et al., 1990) As the absence of Clever-1 only reduced the lymphocyte migration by 50%, one can envisage that other mechanisms or adhesion molecules are possibly involved in this process. Intravital imaging was not feasible in this study since the differentiation between the Clever-1 positive and negative vessels was not feasible. We also found that LPS-induced inflammation downregulated Clever-1 expression on spleen RP sinusoids and its downregulation increased the migration of $CD11b^+/Gr\text{-}1^+$ cells into the spleen in WT mice. Therefore, one can envisage that Clever-1 directly or indirectly regulates the migration of $CD11b^+/Gr\text{-}1^+$ cells into the spleen.

Lymphocyte migration involves both adhesion molecules on the endothelium and its receptor on the lymphocytes. As the receptor for Clever-1 on leukocytes is currently unknown, we decided to indirectly validate the binding of Clever-1 to its receptor on lymphocytes. We incubated affinity-purified Clever-1 with mouse lymphocytes and then evaluated the binding of Clever-1 antibody to the lymphocytes. We found that Clever-1 can directly bind to B-cells and $CD8^+$ T-cells, whereas it does not bind to $CD4^+$ T-cells. These data also support our *in vivo* homing experiments, in which the migration of $CD8^+$ T-cells and B-cells was impaired in the absence of Clever-1, whereas the migration of $CD4^+$ T-cells was comparable. These data also showed a sufficient evolutionary conservation of Clever-1 binding epitopes in humans and mice.

We next aimed to investigate the underlying mechanism for this impaired lymphocyte migration into the spleen. Clever-1 has been shown to directly or indirectly affect the expression of several genes, such as CCL3 and several pro-inflammatory molecules. (Palani et al., 2016; Viitala et al., 2019) Therefore, we reasoned that the absence of Clever-1 might also affect the molecules involved in lymphocyte homing into the spleen. We therefore studied the gene expression of *Cxcl13, Irak4 and Ifi202b* in splenic BECs. All these genes can either directly or indirectly affect leukocyte migration. Our immunohistochemistry analysis of spleen sections validated the downregulation of CXCL13 in Clever-1 KO mice. CXCL13 and its receptor, CXCR5, play an essential role in the migration and localisation of B-cells and a subset of activated and memory T-cells to follicles in the LNs and spleen. (Ansel et al., 2000; Kanemitsu et al., 2005) IFN-ɣ downregulates the expression of CXCL13 at the protein and mRNA levels and subsequently alters the localisation of lymphocytes within the spleen. (Mueller, Hosiawa-Meagher, et al., 2007) On the other hand, silencing Clever-1 by siRNA or blocking its function by antibody has previously been shown to increase

proinflammatory cytokine production, including IFN-ɣ (Palani et al., 2016). Taken together with the expression of IFN-inducible gene *Ifi202b* mRNA in KO splenic endothelial cells, one can envisage that the elevated level of IFN-ɣ in the absence of Clever-1 leads to downregulation of CXCL13, resulting in impaired lymphocyte trafficking into the spleen.

It has been previously shown that embryonic B-cells are unresponsive toward the CXCL13, but gain the responsiveness shortly after birth and form the unique splenic B-cell compartment. (Neely & Flajnik, 2015) This may explain the normal structure of the spleen in KO mice, together with other factors that control the niche size.

Clever-1 is expressed on both afferent and efferent lymphatic vessels and regulates lymphocyte migration into the LNs (Karikoski et al., 2009). Therefore, in this thesis work, we also aimed to investigate the role of Clever-1 in DC migration into the dLNs. We found that Clever-1 is continuously expressed on both the collecting and capillary lymphatic vessels and regulates the migration of DCs into the dLNs. To migrate to LNs via the lymphatic vessels, DCs first need to adhere, roll, and eventually transmigrate into the lymphatic vessels. Our results revealed that while DCs can normally adhere to lymphatic vessels of KO mice, they cannot transmigrate in the absence of Clever-1. Yet, this underlying mechanism remains unknown. Upon their migration to the LNs, DCs do not usually leave the LNs under normal conditions. (Farstad et al., 1997) Thus, it is probable that the role of Clever-1 on efferent lymphatics is limited to regulating lymphocyte egress from the efferent lymphatic vessels.

Since Clever-1 functions as an adhesion molecule, we expected to detect a lower number of leukocytes in the absence of Clever-1 when studying the number of endogenous leukocytes at homeostatic conditions in the LNs. However, we found no significant difference in the total number of leukocytes or resident DCs and a small, but significantly lower number of migratory DCs and B-cells in the LNs of KO mice. In all our studies, we used full KO mice with depleted Clever-1 genes in all of the cells during their entire lifespan. Hence, we reasoned that a compensatory mechanism may have been acquired in KO mice to cover the effect of impaired leukocyte migration in the absence of Clever-1, and that is why we did not observe a dramatic difference in the number of endogenous leukocytes at steady-state.

## 6.2 Lymphocytes migrate into the spleen via the RP (I)

Despite a significant role of the spleen in the adaptive immune response and its similarity to LNs, the molecular mechanism and migratory pattern of leukocytes into spleen has remained a controversial topic. One of the major differences between the spleen and the LNs is the absence of HEV structures in the spleen. Almost 60 years ago, an early study observed that injected Indian ink initially accumulated in multiple

locations of the MZ and to a lesser extent in the WP. Similarly, sequentially tracing radiolabelled lymphocytes at longer time points than 10 minutes revealed that transferred cells were primarily accumulating in and around the MZ, bridging channels and thereafter in the WP. Hence, it was concluded that, lymphocytes mainly enter the spleen via the MZ sinuses in rodents and thereafter migrate to the WP (Figure 11, Panel A). (Mitchell, 1973; Nolte et al., 2002)

In this study, we aimed to further investigate the migratory pattern of lymphocyte migration into the spleen. We found that Clever-1 is exclusively expressed on the RP blood vessels, but absent from WP vessels or MZ sinuses. *Ex vivo* adhesion assays showed that $CD8^+$ T-cells and B-cells specifically bind to RP blood vessels in a Clever-1-dependent manner. The expression pattern of Clever-1 together with *ex vivo* adhesion assays and impaired lymphocyte migration into the spleen led us to the hypothesis that lymphocytes primarily enter the spleen via the RP vasculatures rather than MZ sinuses. To investigate the migratory pattern of lymphocytes in the spleen, we analysed the localization of newly-arrived transferred lymphocytes after 10 minutes and found that transferred lymphocytes preferentially enter the spleen via the terminal arterioles within the RP, rather than the MZ (Figure 11, Panel B). A possible explanation for the contrasting results to the old hypothesis might be the long-time point used in earlier studies to track the transferred cells. Upon entering the spleen, lymphocytes rapidly distribute between different splenic compartments, and therefore longer time-points than 10 minutes might not represent the initial entrance route of lymphocytes. (Brelińska et al., 1984) Consistent with our results, a very recent study also showed that lymphocytes first enter the spleen via the RP vasculature and then migrate through the RP parenchyma and eventually enter the WP via the bridging channels (BCs) (Chauveau et al., 2020).

We also observed a lower number of lymphocytes in the MZ in the absence of Clever- 1. We reasoned that, as lymphocytes first enter the spleen via the RP blood vessels and then migrate towards the MZ and enter the WP, a lower number of lymphocytes in the RP essentially leads to a lower number of lymphocytes in the MZ. In our study, intravital microscopy was not a feasible method since Clever-1 is not expressed uniformly throughout the vascular tree of the RP. Thus, quantifying lymphocyte migration in a Clever-1-dependent manner was not feasible.

Even though it has been well established that lymphocytes use BCs as a corridor for their migration, whether lymphocytes migrate unidirectionally or bidirectionally through the BCs has remained controversial. While some early studies supported the model that lymphocytes use BCs as an exit route to unidirectionally migrate to the RP venous system, others have suggested that lymphocytes use BCs as a transport corridor to migrate between RP and WP bidirectionally. (Bajénoff et al., 2008; Brelińska et al., 1984; Khanna et al., 2007; Mitchell, 1973) As we did not perform intravital imaging, we could not address this question. However, more recently, with

the help of advanced intravital imaging techniques, Chauveau et al. showed that once released into the RP sinuses, T-cells unidirectionally follow a path guided by blood vessels that function as a scaffold for a network of stroma-coated routes, called perivascular T-track (PT-track), towards the WP. They also showed that even though lymphocyte attachment to the PT-track is integrin- and CCR7-independent, these molecules are required for their directional migration along the PT-track to the WP. However, even though Chauveau et al. clearly showed that lymphocyte migration into the WP through the BC is unidirectional, they could not show an exit route for lymphocyte egress from WP (Figure 11, Panel B). (Chauveau et al., 2020) Further studies are needed to determine the exit route of lymphocytes from the spleen.

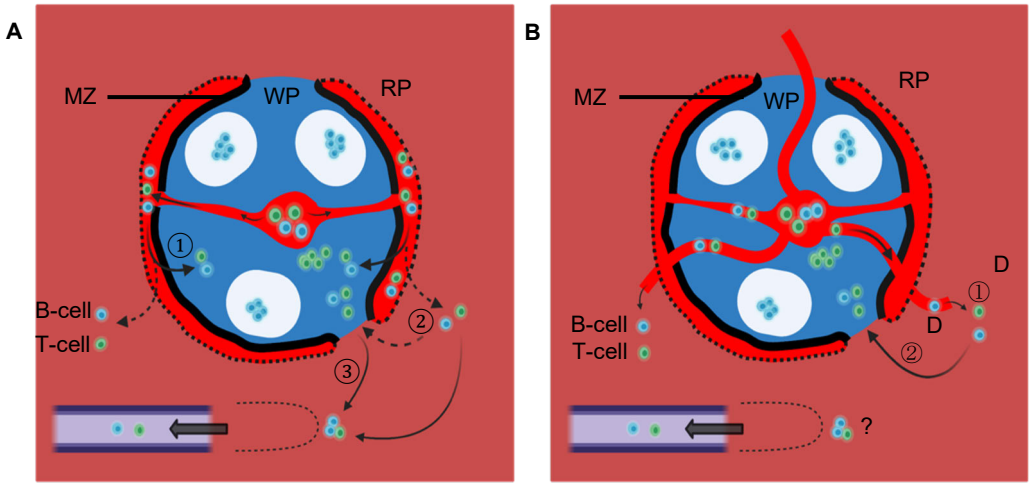


**Figure 11. Possible routes of lymphocyte migration into the spleen and their exit. (A)** A former hypothesis believed that: 1) The majority of lymphocytes first enter the spleen via the marginal zone (MZ) sinuses and then migrate to the white pulp (WP). 2) A minority of lymphocytes can also first enter the red pulp (RP) and migrate to the WP through the bridging channels (BCs). 3) Lymphocytes eventually exit the WP throught the BCs to enter the venous sinuses and return to the blood circulation **(B)** A revised hypothesis suggests that: 1) the majority of lymphocytes first enter the RP from the terminal arterioles. 2) Lymphocytes then migrate unidirectionally into the WP throught the bridging channels. ?) It remains unknown how these cells return into the blood circulation. This figure was created with www.biorender.com.

## 6.3 Clever-1 supports tolerance induction by LECs (II)

DCs in peripheral tissues constantly migrate to the dLNs and carry antigens to present them to T-cells to initiate adaptive immune responses. Impaired DC trafficking to the dLNs has been shown to decrease the antigen-specific immune

response. (Sallusto & Lanzavecchia, 2002) When studying the antigen-specific immune response in KO mice, we reasoned that the observed decrease of DCs would eventually result in lower antigen-specific T-cell responses, if not compensated by other mechanisms. Contrary to our expectation, we observed a more robust antigen-specific immune response in KO mice. In searching for the underlying reasons, we found that DCs in Clever-1 deficient microenvironment expressed higher levels of MHC II molecules. We reasoned that higher MHC II expression by DCs is the possible underlying reason for the increased $CD4^+$ T-cell proliferation in response to ovalbumin in KO mice. However, what regulates the activation of DCs in a Clever-1-deficient microenvironment was still unknown. Therefore, we next aimed to investigate the underlying mechanism of this stronger DC activation in KO mice.

Until recently, it was believed that peripheral lymphatic vessels only direct immune cells and passively transfer antigens from the periphery to the dLNs. However, it has become clear that LECs play an essential role in modulating the immune response. LECs utilize different mechanisms to down-modulate the immune response. For instance, LECs express high levels of several co-inhibitory molecules, such as PD-L1, but lack costimulatory molecules, including CD40, CD80, and CD86. (Dubrot et al., 2014; Rouhani et al., 2015; Tewalt, Cohen, Rouhani, Guidi, et al., 2012) In addition, DCs constantly interact with LECs while migrating along within the lymphatic lumens (Russo et al., 2013), and this DC-LEC adhesive interactions downregulate the expression of the costimulatory molecule CD86 on DCs and reduce the ability of DCs to induce T-cell proliferation. (Podgrabinska et al., 2009) Blocking this interaction induces DC activation and their expression of costimulatory molecules and subsequently restores their ability to induce T-cell proliferation. (Podgrabinska et al., 2009) In addition to a direct interaction, DCs cocultured with LEC-conditioned medium express lower levels of MHC II, CD40 and IL-6. (Christiansen et al., 2016) Consistent with these findings, the migratory DCs from peripheral tissues have a more tolerance-inducing ability than the blood-derived LN-resident DCs. (Idoyaga et al., 2013) Taking these previous findings in consideration, when studying the effect of Clever-1-deficient LECs on the modulation of DCs, we reasoned that Clever-1 on LECs acts as an immunosuppressive molecule suppressing DC activation, and its absence would increase DC activation and T-cell proliferation. Indeed, human DCs cocultured with Clever-1 silenced HDLECs showed higher expression of MHC II, and when cocultured with T-cells in a mixed leukocyte reaction (MLR) assay, they were able to provoke more robust CD4+ T-cell proliferation. These results indicate that Clever-1 deficient LECs are incapable of down-modulating DC activation.

These data were further supported by RNA-seq analysis of LECs isolated from the dLNs of KO and WT mice. KO LECs at steady-state upregulated pathways associated with inflammatory responses compared to their WT controls. Analysing

the genes involved in these pathways revealed that several proinflammatory genes, including *Ilb*, *Csf3*, *Ifi202b*, *S100A9* and *MAdCAM1*, were upregulated in KO LECs. We also studied the gene expression of *Ackr2*, *Csf2*, and *Tlr3* in the inflamed KO LECs. All of these genes can either directly or indirectly affect leukocyte migration, DC maturation and tolerance induction (II: Figure S2D); *Ackr2* is involved in leukocyte migration (Bonavita et al., 2017), *Csf2* regulates the development of inflammatory DC (Greter et al., 2012; Urdinguio et al., 2013), and *Tlr3* is reported to induce tolerance (Fletcher et al., 2010). However, further studies are needed to determine the role of Clever-1 on these genes.

LECs have been shown to directly present peripheral and exogenous antigens on their MHC I and II molecules and induce $CD8^+$ T-cell deletional tolerance and $CD4^+$ T-cell anergy. (Fletcher et al., 2010, 2011; Hirosue et al., 2014; Rouhani et al., 2015; Tewalt, Cohen, Rouhani, & Engelhard, 2012) We found that inflamed Clever-1 KO LECs downregulated the genes associated with antigen presentation. Taken together with the enhanced $CD4^+$ T-cell proliferation, these data suggest that the absence of Clever-1 may also restrict LECs ability to interact with T-cells and induce tolerance. Further studies are needed to determine the role of Clever-1 in T-cell and LECs interactions.

## 6.4 Clever-1 controls the magnitude of the inflammatory response (II)

As the absence of Clever-1 boosts antigen-specific immune responses, we aimed to further investigate its role in a more complex inflammatory model, namely the CHS model, where DC migration as well as DC- and T-cell-activation contribute to an appropriate immune response. The murine model of CHS is induced in two distinct phases: sensitization and elicitation (Figure 12). The sensitization phase is induced by introducing a reactive hapten on the skin, which after percutaneous penetration binds to host proteins and is eventually presented by the MHC molecules of DCs to naïve T-cells in the dLNs. (Bour et al., 1995) The efficacy of the sensitization phase essentially depends on the ability of DCs to present the hapten antigens and generate the hapten-specific effector T-cells in the skin dLNs. (Bennett et al., 2007; Brewig et al., 2009; Honda et al., 2013; Kaplan et al., 2012) It has been previously reported that impaired DC migration in the sensitization phase can reduce the capacity of the immune response to mount a proper CHS response in the elicitation phase. (Del Prete et al., 2004; Sawada et al., 2015) However, as we observed an enhanced antigen-specific immune response in KO mice, we expected a greater CHS response in KO mice than their WT controls. Contrary to our expectation, we observed an attenuated CHS response in KO mice. Since this could not be due to the lower number of hapten-primed T-cells, we reasoned that the possible underlying reasons must be in the elicitation phase.

The elicitation phase is initiated by re-exposure of the same reactive hapten. (Bour et al., 1995) In this phase, memory T-cells rapidly leave the dLNs via the efferent lymphatic vessels, enter the bloodstream and migrate to the inflamed tissue. Also a portion of T-cells that leave the LNs earlier rapidly migrate to the inflamed tissue. (Bour et al., 1995) Clever-1 is upregulated on the inflamed blood endothelium and it regulates the migration of leukocytes to the inflamed tissue. (Karikoski et al., 2009) Clever-1 is also expressed on the afferent and efferent lymphatic vessels and regulates lymphocyte migration via the lymphatics. (Karikoski et al., 2009) Therefore, we reasoned that either impaired egress of lymphocytes from the dLNs, impaired entry to the site of OXA challenged tissue, or both is the possible underlying reason for the lower CHS response in KO mice. Indeed, transferring OXA-primed lymphocytes directly to the blood circulation could restore the CHS response in KO mice partially but failed to fully rescue it. These results suggest that both lymphocyte egress from the dLNs and lymphocyte entry to the inflamed tissue are impaired in the absence of Clever-1 (Figure 12). Indeed, quantification of the transferred OXA-primed lymphocytes confirmed that lymphocyte migration into the OXA-inflamed ears is impaired in KO mice.

During the elicitation phase, effector leukocytes secrete different cytokines to mediate the tissue edema and ear swelling. (Grabbe & Schwarz, 1998) To further seek out the underlying reason for lower CHS response in KO mice, we investigated the levels of effector cytokines in KO mice. Among the effector cytokines, IFN-ɣ and TNF-α are considered to be the most crucial cytokines for the CHS response. (Xu et al., 1996) Neutralizing TNF-α completely diminishes and neutralizing IFN-ɣ partially reduces the CHS response. (Piguet et al., 1991; Saulnier et al., 1995) IL-17 also plays a significant role in both the sensitization and elicitation phases. (Kish et al., 2009) In contrast to the pro-inflammatory cytokines, IL-4 and IL-10, secreted by $CD4^+$ T-cells have a more regulatory effect on the CHS response. (Xu et al., 1996) Indeed, we found that ears from KO mice contain more IL-4 anti-inflammatory cytokines one day after OXA challenge. The inflammatory cytokines, including IFN-ɣ, IL-17, IL-1α and TNF-α, were higher in the KO ears at steady-state but were downregulated after sensitization. Consistent with our RNA-seq data, these findings show that the absence of Clever-1 at steady-state results in a more pro-inflammatory microenvironment, whereas, upon inflammation, it changes to a more anti-inflammatory microenvironment. One can envisage that immune responses are down-modulated in KO mice to maintain homeostasis as a feedback mechanism. Further studies are needed to determine this feedback mechanism.

In addition to T-cells, other subsets of leukocytes are important in the CHS immune response. Upon re-exposure to the hapten, macrophages rapidly develop a cluster with effector T-cells around postcapillary venules to initiate the activation and proliferation of the effector T-cells in an antigen-dependent manner. (Natsuaki

et al., 2014) The importance of developing these perivascular leukocyte clusters during the initiation and extension of the immune response against the hapten is evidenced in the animal model of CHS. (Natsuaki et al., 2014) In addition, depletion of macrophages has been shown to attenuate DC migration as well as DC-T-cell clustering and subsequently impairs the CHS response. (Natsuaki et al., 2014) On the other hand, Clever-1 has been shown to be expressed on a subset of immunosuppressive macrophages and its expression has been associated with an immunosuppressive microenvironment. (Viitala et al., 2019) Therefore, we investigated whether Clever-1-deficiency on macrophages affects the CHS response or DC migration. CD11c-YFP chimeric mice showed that reconstituting the KO mice with CD11C-YFP bone marrow cells could not rescue the CHS response or DC migration, suggesting that the impaired CHS response and DC migration is independent of the lack of Clever-1 on macrophages.

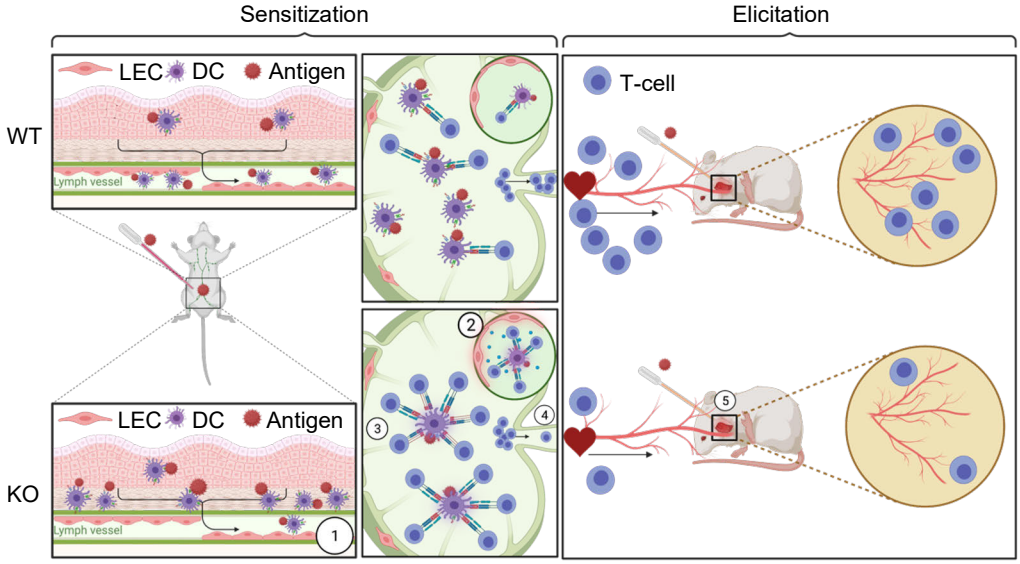


**Figure 12. The role of Clever-1 in different phases of the CHS model. 1)** During the sensitization phase, dendritic cells (DCs) cannot efficiently transmigrate into the Clever-1-deficient lymphatic vessels. **2)** Clever-1 deficient lymphatic endothelial cells (LECs) are incapable of downmodulating DC activation and T-cell proliferation. **3)** Therefore, a lower number of migratory DCs are able to activate a greater number of T-cells in the Clever-1 KO microenvironment. **4)** The absence of Clever-1 on the efferent lymphatics impairs the egress of lymphocytes from the draining lymph nodes (dLNs). **5)** during the elicitation phase, the absence of Clever-1 on the inflamed blood endothelium impairs the migration of T-cells into the inflamed ear. This figure was created with www.biorender.com.

# 7 Conclusion

This study describes a central role of Clever-1 in regulating lymphocyte and DC trafficking into peripheral lymphoid tissues. Furthermore, we identified a new route for lymphocyte entry into the spleen, which changed the long-standing perception that these cells enter the spleen via the marginal zone or the vasculature in the white pulp. The discovery of this new route sheds light on the filtering function of the spleen, where antigens can be recognized by immune cells and activate the adaptive immune response. This may further enable us to directly target the red pulp resident cells for novel therapeutic purposes.

Moreover, we showed a specific role of lymphatic Clever-1 in the regulation of adaptive immune responses. These results answer some fundamental questions on how lymphatic endothelium orchestrates immune responses. Our results may ultimately be used to open new therapeutic strategies for different diseases, where appropriate modulation of immune responses is needed. The specific findings of this thesis work can be summarized in the following points:

1- Lymphocytes preferentially enter the spleen via the vessels in the red pulp rather than the marginal sinus or white pulp vasculature

2- Clever-1 is exclusively expressed on the blood endothelial cells of red pulp and regulates lymphocyte migration into the red pulp

3- Clever-1 is expressed on the peripheral lymphatic vessels and regulates the transmigration of dendritic cells into the lymph nodes

4- Clever-1 on lymphatic endothelial cells dictates the magnitude of the adaptive immune response by regulating the expression of co-stimulatory molecules on dendritic cells

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