UNIVERSITY OF TURKU

PLVAP AS THE GATEKEEPER OF ENDOTHELIAL CELL PERMEABILITY AND LEUKOCYTE MIGRATION

Norma Jäppinen

TURUN YLIOPISTON JULKAISUJA – ANNALES UNIVERSITATIS TURKUENSIS
SARJA – SER. D OSA – TOM. 1445 | MEDICA – ODONTOLOGICA | TURKU 2019

UNIVERSITY OF TURKU

PLVAP AS THE GATEKEEPER OF ENDOTHELIAL CELL PERMEABILITY AND LEUKOCYTE MIGRATION

Norma Jäppinen

TURUN YLIOPISTON JULKAISUJA – ANNALES UNIVERSITATIS TURKUENSIS
SARJA - SER. D OSA – TOM. 1445 | MEDICA – ODONTOLOGICA | TURKU 2019

University of Turku

Faculty of Medicine
Institute of Biomedicine
Medical Microbiology and Immunology
Turku Doctoral Programme of Molecular Medicine

Supervised by

Academician MD, PhD, Sirpa Jalkanen
Institute of Biomedicine
MediCity Research Laboratory
University of Turku
Turku, Finland

Professor, MD, PhD Marko Salmi
Institute of Biomedicine
MediCity Research Laboratory
University of Turku
Turku, Finland

Docent, PhD, Pia Rantakari
Institute of Biomedicine
University of Turku
Turku, Finland

Reviewed by

Professor, MD, PhD, Risto Renkonen
Department of Bacteriology and Immunology
Transplantation Laboratory
University of Helsinki
Helsinki, Finland

Docent, PhD, Marja Mikkola
Institute of Biotechnology
University of Helsinki
Helsinki, Finland

Opponent

Professor, MD, PhD, Mika Rämet
PEDEGO Research Unit
University of Oulu
Oulu, Finland
Laboratory of Experimental Immunology
University of Tampere
Tampere, Finland

The originality of this publication has been checked in accordance with the University of Turku quality assurance system using the Turnitin OriginalityCheck service.

ISBN 978-951-29-7780-2 (PRINT)
ISBN 978-951-29-7781-9 (PDF)
ISSN 0355-9483 (Print)
ISSN 2343-3213 (Online)
Painosalama, Turku, Finland 2019

Perheelleni

UNIVERSITY OF TURKU
Faculty of Medicine
Institute of Biomedicine
Medical Microbiology and Immunology
NORMA JÄPPINEN: PLVAP as the Gatekeeper of Endothelial Cell Permeability and Leukocyte Migration
Doctoral Dissertation, 214 pp.
Turku Doctoral Programme of Molecular Medicine
October 2019

ABSTRACT

Endothelial cells (EC) line the lymphatic and vascular vessels of the body, forming a barrier between the lumen and surrounding tissue. ECs actively coordinate the exchange of migrating cells, fluids and molecules thus regulating tissue homeostasis. Correctly working endothelium is also important during the embryonic development, as progenitor cells have tight time windows to reach and colonize target tissues. Failures to do so lead to perturbed cell composition and lasting adverse effects in the adulthood. In the blood endothelium, plasmalemma vesicle-associated protein (PLVAP) forms diaphragms on specific plasma membrane structures (transendothelial channels, fenestrae and caveolae) and has been showed to regulate the EC permeability in blood vessels. However, the role of PLVAP in the lymphatic endothelium is not characterized, nor its role in the immune system during development.

The aim of this thesis was to use in vivo and in vitro -methods to investigate endothelial cells in the lymph node and liver. The results of this thesis show that PLVAP is unexpectedly expressed in distinct lymphatic ECs of the lymph node and there, PLVAP regulates the antigen and lymphocyte entry to the lymph node. During development PLVAP expression is crucial for migration of macrophage progenitors and the emerging of a F4/80^{Hi} tissue-resident macrophage population in several organs. In mammary gland, the F4/80Hi macrophages form a major population of the steady state macrophage pool and that their unique functions cannot be performed by other macrophage types.

KEYWORDS: Endothelium, PLVAP, lymph node, cell traffic, tissue specific macrophage, mammary gland

TURUN YLIOPISTO
Lääketieteellinen Tiedekunta
Biolääketieteen Laitos
Lääketieteellinen mikrobiologia ja immunologia
NORMA JÄPPINEN: PLVAP:n vaikutus endoteelisolupermeabiliteettiin ja valkosolumigraatioon
Väitöskirja, 214 s.
Turku Doctoral Programme of Molecular Medicine
syyskuu 2019

TIIVISTELMÄ

Kehon veri- ja imusuonistoa vuoraa endoteelisoluista koostuva kerros, joka toimii aktiivisena rajapintana suonten sisätilan ja ympäröivien kudosten välillä. Endoteelisolut suodattavat lävitseen soluja, nesteitä sekä molekyylejä ja säätelevät näin kehon fysiologista tasapainoa. Endoteelisolujen toiminta on tärkeää aikuisten kudosten homeostaasin ylläpidon säätelyssä, mutta myös sikiönkehityksen aikana ohjaamassa solujen esiasteiden kulkua kohdekudoksiinsa. Plasmalemma vesicle-associated protein (PLVAP) on veriendoteelisolujen pinnalla ilmentyvä proteiini, joka muodostaa siivilämäisiä suodattimia endoteelisoluihin. PLVAP:n on aikaisemmin osoitettu vaikuttavan endoteelisolujen läpäisevyyteen verisuonistossa.

Tämän väitöskirjan tarkoituksena on tutkia in vivo ja in vitro-metodeja käyttäen imusolmukkeen ja maksan endoteelisoluja ja selvittää, kuinka PLVAP-riippuvainen endoteelisolupermeabiliteetti säätelee soluliikennettä ja miten soluliikenteen häiriintyminen sikiönkehityksen aikana vaikuttaa myöhemmin kudoksissa.

Väitöskirjatutkimuksessa selvitettiin, että PLVAP esiintyy odottamattomasti imusolmukkeen sinuksen endoteelisoluissa, joissa se säätelee antigeenien ja solujen pääsyä imusolmukkeeseen. Lisäksi selvisi, että sikiökautisessa maksassa PLVAP säätelee makrofagien esiasteiden pääsyä verenkierron kautta kohdekudoksiinsa. Näytimme, että normaalitilassa rintakudoksen makrofagipopulaatio koostuu merkittävin osin sikiöperäisistä kudosmakrofageista, joilla on erityistehtäviä, joita muut makrofagit eivät pysty suorittamaan.

AVAINSANAT: Endoteeli, PLVAP, imusolmuke, soluliikenne, kudosspesifiset makrofagit, rintakudos

Table of Contents

Abbreviations

AGM	Aorta-Gonad-Mesonephros
APC	Antigen Presenting Cell
BABB	Benzyl Alcohol and Benzyl Benzoate
BEC	Blood Endothelial Cell
BM	Basement membrane
BrdU	Bromodeoxyuridine
BSA	Bovine Serum Albumin
CCL	Chemokine Ligand
CCR	Chemokine Receptor
cDNA	Complementary Deoxyribonucleic acid
CFSE	Carboxyfluorescein Succinimidyl Ester
CSF1	Colony Stimulating Factor 1
CSF1R	Colony Stimulating Factor 1 Receptor
CTLA4	Cytotoxic T lymphocyte antigen 4
CyTOF	Cytometry by Time Of Flight
DAPI	4′,6-diamidino-2-phenylindole
DC	Dendritic Cell
DNA	Deoxyribonucleic acid
E	Embryonic Day
EC	Endothelial Cell
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediaminetetraacetic Acid
EYFP	Enhanced Yellow Fluorescent Protein
EMP	Erythro-Myeloid Progenitor
FRC	Fibroblastic Reticular Cell
GFP	Green Fluorescent Protein
HEV	High Endothelial Venule
HLA	Human Leukocyte Antigen
HRP	Horseradish peroxidase
HSC	Hematopoietic Stem Cell
ICAM	Intercellular Adhesion Molecule-1

IFN γ	Interferon Gamma
IL	Interleukin
i.p	Intraperitoneal
i.v	Intravenous
LDL	Low Density Lipoprotein
LEC	Lymphatic Endothelial Cell
LPS	Lipopolysaccharide
MDP	Macrophage Dendritic Cell Precursor
MFI	Mean Fluorescence Intensity
MHC	Major histocompatibility complex
MP	Myeloid Precursor
mRNA	Messenger Ribonucleic Acid
OCT	Optimal Cutting Temperature Compound
OVA	Ovalbumin
PAL-E	Pathologische Anatomie Leiden-Endothelium
PBS	Phosphate Buffered Saline
PD1	Programmed Cell Death-1
PFA	Paraformaldehyde
PLA	Proximity Ligation Assay
PLN	Peripheral Lymph Node
PLVAP	Plasmalemma Vesicle Associated Protein
qPCR	Quantitative Polymerase Chain Reaction
s.c	Subcutaenous
SCS	Subcapsular Sinus
SDS-PAGE	SodiumDodecylSulfate–Polyacrylamide Gel Electrophoresis
TBS	Tris Buffered Saline
TEB	Terminal End Bud
TEC	Transendothelial Channel
TNFα	Tumor Necrosis Factor Alpha
t-SNE	T-distributed Stochastic Neighbor Embedding
VEGF	Vascular Endothelial Growth Factor
WT	Wild Type
YFP	Yellow Fluorescent protein
YS	Yolk Sac

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 Pia Rantakari*, Kaisa Auvinen*, Norma Jäppinen, Maria Kapraali, Joona Valtonen, Marika Karikoski, Heidi Gerke, Imtiaz Iftakhar-E-Khuda, Johannes Keuschnigg, Eiji Umemoto, Kazuo Tohya, Masayuki Miyasaka, Kati Elima, Sirpa Jalkanen & Marko Salmi. The endothelial protein PLVAP in lymphatics controls the entry of lymphocytes and antigens into lymph nodes. *Nat Immunol*. 2015, doi: 10.1038/ni.3101

II Pia Rantakari*, Norma Jäppinen*, Emmi Lokka, Elias Mokkala, Heidi Gerke, Emilia Peuhu, Johanna Ivaska, Kati Elima, Kaisa Auvi-nen & Marko Salmi. Fetal liver endothelium regulates the seeding of tissue-resident macrophages. *Nature. 2016*, doi: 10.1038/nature19814

III Norma Jäppinen, Inês Félix, Emmi Lokka, Sofia Tyystjärvi, Anne Pynttäri, Tiina Lahtela, Heidi Gerke, Kati Elima, Pia Rantakari & Marko Salmi. Fetal-derived macrophages dominate in adult mammary glands. Nat Commun. 2019, doi: 10.1038/s41467-018-08065-1.

*equal contribution

1 Introduction

The wellbeing of an individual largely depends on the ability to maintain efficiently working defense systems in the body, be it during the physiological steady state or under threats during pathological conditions like inflammation or cancer. Well-functioning communication between organs, facilitated by vasculature, is the cornerstone of immunity. The network of vessels provides a highway for the signals from the periphery to be distributed to the vital lymphoid organs where they are interpreted and reacted to accordingly. The vasculature can be divided to two distinct parts, the blood vasculature and the lymphatic vasculature. The two are intertwined, and have structural similarities among the dividing factors. The innermost layer of both vasculatures consists of endothelium, a cell layer that not only acts as a barrier between the lumen of the vessel and surrounding tissues, but also actively regulates the permeability of the vessels. Disturbances in the vessel permeability can lead to disturbed exchange of fluids, molecules and cells. (Cleaver and Krieg, 2010, Escobedo and Oliver, 2016) The regulation of cell traffic is especially important during embryogenesis, which is a precisely regulated cascade of events within a narrow time window. (Melton and Cleaver, 2003) Perturbations in cell trafficking caused by incorrectly working endothelium during development can lead to lasting effects on the cellular composition and function of tissues.

Plasmalemma Vesicle Associated Protein (PLVAP) is an endothelial cell specific protein which has been shown to regulate cell traffic and the permeability of fenestrated blood endothelium. (Stan et al. 2012) However, little is known about PLVAPs role in the lymphatics and the cell traffic across the lymphatic endothelium. The research projects in this thesis show for the first time that PLVAP is also expressed in the lymphatic endothelial cells (LECs) of the lymph node and there it regulates the permeability of lymphatic endothelial cells of the subcapsular sinus. During the study of the immunological role of PLVAP, it became evident that the PLVAP deficient mice have major perturbations in macrophage populations in several tissues. The emergence of tissue-resident macrophages occurs during embryogenesis, in a process which involves cell migration across the liver sinusoidal endothelium. (Rafii et al. 1997) The molecules

which govern the migration of the fetal macrophage precursors were unknown, thus we hypothesized a role for PLVAP as it affects permeability in several other tissues. The results showed that the expression of PLVAP during development is necessary for the emergence of F4/80^{Hi} tissue-resident macrophages in adulthood. Finally, a closer look at the mammary gland was taken, since the macrophage dependent ductal development was clearly perturbed in the *Plvap*$^{-/-}$ mice. It was shown that the majority of macrophages in the adult mammary gland originate from the fetal era and that the tissue-resident macrophages have a distinct phenotype and function in comparison to monocyte-derived macrophages. Detailed information about tissue macrophage composition is crucial for the development of new treatment strategies that rely on modulation of endogenous immune cells.

2 Review of the Literature

The immune system is an intricate combination of specialized subsystems. This literature review will discuss the development and functions of blood and lymphatic vasculature, lymph nodes, macrophages and mammary gland. The role of endothelium in cell and molecule migration at steady state and the role of PLVAP in the permeability of endothelial cells will also be discussed.

2.1 The immune system

The immune system is a system of cells and organs working together in order to protect against a disease and maintain the host homeostasis. The body comes in contact with a vast amount of both new and previously encountered antigens daily. Bacteria, viruses, parasites and fungi, or a part of them or their products act as antigens that need to be recognized from host cells and, if necessary, destroyed efficiently. To achieve this, the body has two systems that work together to protect the host: the innate immunity and the adaptive immunity. The innate response is rapid, but also less specific and somewhat explosive, whereas the adaptive immunity reacts more slowly but counters with specific responses and an immunological memory which helps fight a reoccurring infection. (Iwasaki, Medzhitov 2015, Wood 2015)

The body has several tiers of protection against pathogens. Pathogen entry into the body is limited by the physical barrier of the intact skin on the outside and mucosal barriers on the inside of the body, in addition to commensal bacteria and resident immune cells in both barriers. (Naik et al. 2012) If the antigen has been able to find its way to the body, the innate immunity is the first responder. The initiation of first immune response relies on recognition of highly conserved pathogen-associated molecular patterns which are present in large groups of micro-organisms. (Janeway 1992) The first reaction to infection or injury is usually inflammation, driven by local macrophages, fibroblasts, mast cells, and dendritic cells (DCs) and accompanied by circulating leukocytes, including neutrophils and monocytes. (Newton, Dixit 2012) A more elaborate and targeted response occurs shortly after, once the antigen reaches the lymph node and is presented to T cells with the help of antigen presenting cells (APCs). Activated T cells differentiate

into CD4+ T helper cells which can assist other T cells in differentiating into cytotoxic T cells which have the ability to kill other cells. T helper cells can also promote B cell responses by helping B cells become plasma cells which produce large quantities of antibodies. Some of the B cells undergo affinity maturation and differentiate into memory cells which remain in the body and can start the production of higher affinity antibodies quickly if the same antigen is encountered a second time. The APCs, T cells and B cells work together to produce an appropriate adaptive immune reaction. (Wood 2015)

Even though inflammation is often the first appropriate reaction to many external or internal threats, like pathogens and tissue injury, it needs to be carefully controlled. Correctly working immunity creates a proportional response to antigens or infection caused by them and is able to curb the inflammation when the threat subsides. (Koh, DiPietro 2011) Problems in controlling the immune response can lead to chronic inflammation, autoimmune diseases or immunodeficiency.

Chronic inflammation is linked to a heightened risk for cardiovascular disease (Ridker et al. 1997) and type 2 diabetes (Duncan et al. 2003), both major health issues worldwide. Autoimmune diseases are the result of the immune system mistakenly attacking healthy tissues and destroying autologous cells. These diseases, such as multiple sclerosis, Crohn's disease and autoimmune thyroiditis are becoming more prevalent in the Western societies, perhaps due to environmental factors. (Lerner et al. 2016) In addition to the diseases caused by an over aggressive immune system, the lack of immune responses can be even more dangerous and often lethal. Immunodeficiency caused by genetic mutations (Buckley et al. 1999) or the human immunodeficiency virus (Autran et al. 1997) leads to loss of immune cells or their function and severely hampers the ability to fight infections. Thus, modulation of the immune system is warranted as the diseases caused by overly active immune system and immunodeficiency not only cause pain and discomfort but also predispose the patients to other diseases and cancer. (Grulich et al. 2007, Park et al. 2010)

Curbing the immune responses in order to control extreme inflammation or autoimmune reactions relies on immunosuppressive medication. Drugs in the glucocorticoid family (Croxtall et al. 2002) and non-steroidal drugs and biologics (Pato et al. 2011) are commonly used for the inhibition of lymphocyte activity. The immune system can be activated before the onset of a disease through immunization, commonly achieved with vaccinations. Vaccinations, often combined with a response enhancing adjuvant (Mosca et al. 2008), activate the immunological memory and provide a relatively long term protection against certain diseases later in life. (Hammarlund et al. 2016) Boosting the immune activity in immunosuppressive conditions can be achieved by a combination of drugs to decrease the viral load and allow immune cell recovery (Autran et al.

1997) or by hematopoietic stem cell gene therapy (Clarke et al. 2018). Despite the various existing methods to modulate the immune system, new approaches are needed, because many of the current methods are aggressive and have adverse effects due to non-specificity of drug distribution and the development of resistance. (Pato et al. 2011, Wilson et al. 2019)

The protective and homeostasis maintaining effects of the immune system often relies on the presence of immune cells. For the purpose of surveilling the body and reaching the site of action, the immune cells must travel using the blood and lymphatic vessels which act as highways from tissue to another. The next sections review the development of the two important vessel systems and the inner lining cell compartment of the vessels, the endothelial cells, with particular in-sight to the endothelial protein PLVAP.

2.1.1 The cardiovascular and lymphatic vasculature

The circulatory system has developed to offer nutrients and oxygen to every cell in the body, to transport waste from the tissues and to facilitate cell traffic throughout the body. The cardiovascular and lymphatic vasculatures circulate blood and lymph respectively, to ensure that the tissues receive nutrients and also to simultaneously facilitate immune surveillance. Blood vasculature distributes oxygenated blood from the arteries to the vessels which sprout from the larger veins, finally reaching the small capillaries. Blood is circulated back towards the lungs to release the carbon dioxide and re-oxygenate the blood. (Cleaver and Krieg 2010) Similarly to the blood vasculature, the lymphatic vessels traverse most of the body. Even the central nervous system, which was thought to be devoid of lymphatic vasculature, has been shown to be drained by lymphatic vessels in certain areas, in addition to the local microglia-dependent drainage. (Aspelund et al. 2015, Petrova and Koh 2018) The lymphatic capillaries collect interstitial fluid, antigens and activated lymphocytes into larger lymphatic vessels, which transport the lymph to the thoracic duct, which empties into the central veins. In addition to lymph drainage, the lymphatics contribute to lipid metabolism. (Wang et al. 2017)

Cells and molecules in the vessels must enter and exit the lumen at suitable points to reach their destination within the tissues. Intra- and extravasation cascades of lymphocytes are complex multistep processes which involve several proteins and interaction between the lumen facing endothelium and the migrating lymphocytes. (Vestweber 2015) Structurally, the blood and lymphatic vessels are composed of an inner lining of endothelial cells and varying degrees of basement membrane (nonexistent in some parts). The vessels are surrounded by different widths of smooth muscle cells depending on the vessel type and finally are wrapped in elastic tissue and fibrous connective tissue. The contracting smooth

muscle cells and luminal valves in the veins and bigger lymphatic vessels help the blood and lymph flow going to the right direction. Blood is pumped in a closed circuit system that is powered by the heart, while lymph seems to flow in a more passive manner due to body movement and respiration. (Cleaver and Krieg 2010, Jones and Min 2011, Wang et al. 2017)

2.1.1.1 Development of blood and lymphatic vasculature

The cardiovascular system is one of the first functional organ systems to develop in the embryo, understandably so because the system provides nutrients and transports cells and signals within the developing fetus and thus enables the growth of an individual. (Ferrara et al. 1996) In the extra embryonic mouse yolk sac, blood islands form from mesoderm-derived hemangioblasts. Hemangioblasts give rise to endothelial cell precursors, angioblasts, and hematopoietic precursors. The hematopoietic precursors give rise to primitive red blood cells by E8.0 in mice. Simultaneously, the aorta and the heart tube form in the embryo proper without contact with hematopoietic cells. The angioblasts in the yolk sac produce endothelial cells in response to local cues produced by the mesoderm, resulting in the growth of the blood islands which then fuse together, forming the rudimentary vasculature (primary vascular plexus). The endothelial cells proliferate and form new vessels by sprouting from the existing plexus in a process termed angiogenesis. Endothelial proliferation and angiogenesis is often prompted by the vascular endothelial growth factor (VEGF) signaling, mediated by the receptors VEGFR1 (Flt-1) and VEGFR2 (Flk-1) on the endothelial cells. (Ferrara et al. 1996) Functional circulation is achieved at E8.5, when the yolk sac and embryo proper vasculature are connected, although it takes two more days for the circulation to be fully functional. (McGrath et al. 2003) Finally, the vessels mature to be able to support the increasing blood flow. The endothelial cells synthesize a thicker basement membrane and recruit vascular wall components such as pericytes and smooth muscle cells to stabilize the vessels. Later, vessels develop different characteristics in response to endothelial cell interaction with the surrounding cells, enabling the vessels to transform into arteries, veins and smaller vessels like venules and capillaries according to the tissue demands. Advanced techniques have revealed that several organs such as liver, lung, pancreas, stomach/intestine, and spleen also have hemangioblasts, which contribute to the vasculogenesis (the formation of blood vessels, without sprouting from an existing vessel) within the organ. (Risau et al. 1988, Risau and Flamme 1995, Shalaby et al. 1995, McGrath et al. 2003, Cleaver and Krieg 2010)

In adult tissues, vasculogenesis is rare. The need for increased blood supply is usually met with new blood vessels sprouting from the existing ones (angiogenesis)

in response to tissue signals, as opposed to *de novo* formation of blood vessels. VEGF and angiopoietins 1 and 2 are important for the correct migration and maturation of the sprouting blood vessels. (Hattori et al. 2001, Lim et al. 2004)

The development of lymphatic vasculature occurs simultaneously with vasculogenesis, but is regulated by different signaling. The majority of lymphatic vessels arise from blood vessels, by sprouting from $Prox1^+$ endothelial cells during the early development around E10. (Wigle, Oliver 1999, Martinez-Corral et al. 2015, Escobedo, Oliver 2016) In addition to budding from venous endothelial cells, recent research has revealed that some lymphatic endothelium originates from a nonhematopoietic precursor, the exact cell type of the lymphatic endothelial cell (LEC) progenitor however is not clear. (Martinez-Corral et al. 2015) Once established, the primary lymphatic vessels continue to grow and develop into initial, blind ended lymphatic vessels, larger smooth muscle cell coated collector vessels with valves and finally into lymphatic veins. (Wang et al. 2017)

2.1.1.2 Endothelial cells

Both the blood and lymphatic vasculature are lined with a layer of endothelial cells. As described above, the endothelium plays a pivotal role in the development of new vessels by orchestrating the vessel formation by cell-cell interactions and cytokine production. In adulthood, the endothelial cells remain a dynamic interphase between the cells and molecules in the vessel lumen and the surrounding tissue. Endothelial cells have a heterogeneous gene profile (Chi et al. 2003), however, they perform similar functions in many tissues. Endothelium participates in cell migration by expressing adhesion molecules like intercellular adhesion molecule-1 (ICAM) and AOC3 and producing chemokines. (Stolen et al. 2005, Millán et al. 2006) They also control vasodilation by producing substances to constrict or dilate the vessel and even have the ability to phagocytose. (Ingerman-Wojenski et al. 1981, Dini et al. 1995, Anselmo and Mitragotri 2017) In the lymph node, the endothelium is important for induction of peripheral tolerance, as the LECs are able to present endogenous antigens directly to T cells. (Cohen et al. 2010)

In addition to their secretory and interactional functions, endothelium can be categorized based on the morphology into continuous, discontinuous or fenestrated endothelium (**Figure 1A**). The different endothelial types differ vastly in their permeability. The need for certain type of endothelium highly depends on the vessel size, location and function, therefore all types of endothelium can be found even within one organ, such as the different compartments of the kidney. (Risau 1998) The endothelial type can also experience changes brought on by developmental maturing. During early development, when the liver is an active

hematopoietic organ, the fenestrated liver endothelium is overlaid with diaphragms, but the diaphragms are not found in adult liver. (Stan 2007)

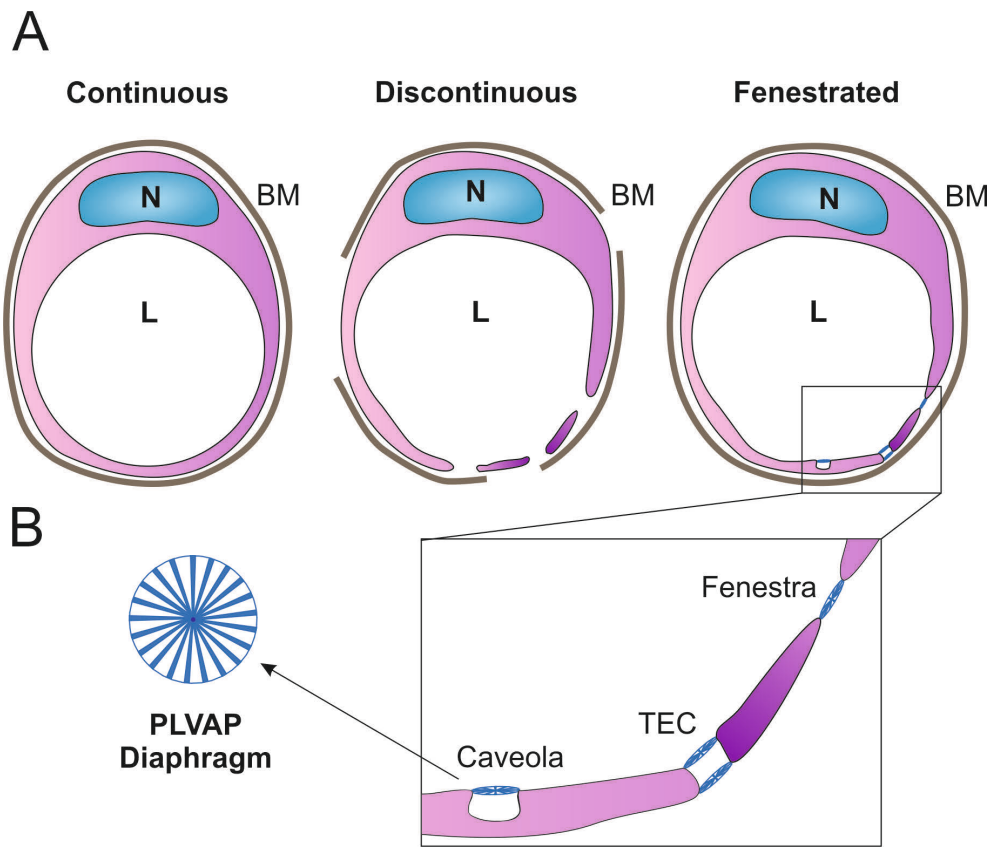

Figure 1. A schematic picture of continuous, discontinuous and fenestrated endothelium (A). Plasma membrane structures caveola, transendothelial channel (TEC) and fenestra with PLVAP diaphragms are pictured in the insert (B). BM= basement membrane, N= nucleus, L= lumen.

Continuous endothelium is characterized by an uninterrupted layer of endothelial cells, where the endothelial cells are joined together by tight, zipper-like junctions, accompanied by a continuous basement membrane. This type of endothelium is typical for veins, arteries and collecting lymphatics and is the most non-permeable endothelium type. The exchange of molecules through the cell is facilitated by vesicle transport. (Predescu et al. 1997, Baluk et al. 2007, Aird 2012)

Small capillaries are often lined with **discontinuous endothelium**. The typical features of discontinuous endothelium are poorly formed basement membrane and the lack of tight junctions between endothelial cells. Discontinuous endothelium is the most permissive of endothelial types and is often found in capillaries of tissues

with extensive molecular traffic such as the liver and bone marrow. (Streeten, Brandi 1990, Risau 1995, Aird 2012, Stan et al. 2012)

Fenestrated endothelium is characterized by transcellular pores which are overlaid with diaphragms. The basement membrane however is continuous. Fenestrated endothelium can be found in organs that are involved in secretion or filtration such as the endocrine glands and intestinal mucosa. (Streeten, Brandi 1990, Aird 2012)

Structures such as caveolae, transendothelial channels (TECs) and fenestrae can be found on the endothelium plasma membrane. These structures are usually found in the peripheral part of the endothelial cell and they are important for the transcellular (through the cell) passage of macromolecules and migration of cells. Caveolae are invaginations of the plasma membrane, coated by caveolin-1, which is an essential protein for the assembly of caveolae. (Razani et al. 2001) Caveolae facilitate the uptake and transport of molecules, such as albumin, from the blood to the tissue. (Schubert et al. 2001) Unlike caveolae, TECs and fenestrae create a direct route through the endothelial cells. Fenestrae are circular openings of the endothelial cell, which often organize in groups called sieve plates. (Loannidou et al. 2006) Depending on the tissue, the aforementioned plasma membrane structures are selectively covered with stomatal diaphragms. (Stan, Kubitza et al. 1999)

So far, only one protein, Plasmalemma Vesicle Associated Protein (PLVAP), has been reported to participate in the formation of the diaphragms. PLVAP diaphragms have been shown to regulate the endothelial cell permeability in fenestrated blood vasculature. The following section will focus on the biology and endothelial functions of PLVAP.

2.1.1.3 Plasmalemma Vesicle Associated Protein (PLVAP) in vasculature

Plasmalemma Vesicle Associated Protein (PLVAP, PV-1 or MECA-32 antigen and in humans PAL-E or FELS) is an endothelial cell specific protein which has only been reported in the blood vessel endothelium and thus widely used as a blood endothelium specific marker. Electron microscopy studies show that PLVAP forms cartwheel like diaphragms on the plasma membrane of endothelial cells. The average diameter of the diaphragm is 60-70 nm and the sieve is composed of fibrils that are conjoined by a central knob, forming openings with the arc width of 5-6 nm. PLVAP is the only known component of the diaphragms and is necessary for their formation. (Bearer, Orci 1985, Rhodin 1962, Stan et al. 2012). Diaphragms are found on endothelium surface structures such as TECs, caveolae and fenestrae **(Figure 1B)** but are notably missing in the intercellular junctions. (Stan, Kubitza et al. 1999, Stan, Ghitescu et al. 1999) The caveolae and transendothelial structures

form independently of PLVAP and are often encountered in tissues with extensive molecular traffic between bloodstream and tissue, such as the liver and endocrine glands, suggesting a role for the fenestrae in the cell traffic and molecule exchange. (Stan et al. 2012) In *in vitro* setting, PLVAP diaphragms are needed for the formation of correctly organized fenestrae and sieve plates and the fenestra formation can be stimulated with VEGF. (Loannidou et al. 2006) In the absence of caveolae, TECs and fenestrae, PLVAP is rapidly internalized, which would suggest that forming the diaphragm is the only role for PLVAP. (Tkachenko et al. 2012)

PLVAP has been extensively studied for decades and the distribution of *Plvap* expression has been mapped in rat (Hnasko, McFarland et al. 2002), mouse and human (normal and neoplastic) tissues (Stan et al. 2001, Strickland et al. 2005). PLVAP mRNA can be found in mouse embryo as early as E7.5, hinting at the fact that PLVAP may be important for normal embryonic development. Expression of *Plvap* mRNA is highest in tissues with extensive traffic between blood and tissues, such as the lung and kidney and little to no expression was detected in the brain and testis. (Stan et al. 2001)

Precious structural data about PLVAP diaphragms have been acquired from *in vitro* studies, but because endothelial permeability is a complex phenomenon, *in vivo* models are necessary to study the role of PLVAP. Mouse and human *Plvap* mRNA distributions show only quantitative differences, which has corroborated the use of mouse models in PLVAP studies. (Stan et al. 2001) Deletion of the gene of interest is a common approach to study the role of a specific gene, thus knock out mouse models of *Plvap* have been developed in a few research groups. The $Plvap^{-/-}$ mice do not survive on a pure breed genetic background and the models are slightly different in regard to their mixed genetic background, but they all show the same hallmarks of PLVAP deficiency: embryonic lethality, blood lipid perturbations and leaky blood vessels. (Herrnberger et al. 2012, Stan et al. 2012) Recently, perturbations in the maintenance of a subpopulation of B cells in the peritoneum and spleen of $Plvap^{-/-}$ mice have been described. (Elgueta et al. 2016) The severe phenotype of the $Plvap^{-/-}$ mice highlights the importance of PLVAP for normal homeostasis.

PLVAP diaphragms in fenestrated blood endothelium regulate permeability by acting as physical sieves. They filter small molecules from the plasma, but leave in sizeable (>70 kDa) proteins. (Stan et al. 2012) In addition to passive filtering, PLVAP diaphragms have also been shown to interact with several molecules and chemokines. Molecules like Neuropilin-1 (Keuschnigg et al. 2012), VEGF (Strickland et al. 2005) and heparin (Hnasko et al. 2002) interact with PLVAP *in vitro*, but the biological function of these interactions have not been elucidated.

2.1.1.4 PLVAP/PAL-E in humans

In humans, the monoclonal antibody PAL-E (Patologische Anatomie Leiden Endothelium) was discovered in the 1980s as a new staining reagent for endothelium. (Schlingemann et al. 1985) In non-pathological tissues, PAL-E was reported to stain the endothelium in the capillaries, sinusoids and small and medium sized veins. The antibody has been useful to discriminate between vascular and lymphatic endothelium, as no signal seems to be detected in the lymphatic ducts. In 2005 it was revealed that PAL-E:s target is actually PLVAP (Niemelä et al. 2005) and the discovery was further solidified by Keuschnigg et al. in 2012 (Keuschnigg et al. 2012).

Only rare cases of *Plvap* gene mutations in humans have been reported, probably because the effects of complete loss of *Plvap* function are so severe that the development of an affected individual stops very early. So far, only two cases of nonsense mutations and two cases of missense mutations have been reported in patients with protein-losing enteropathy. The effects of the missense mutations are milder, but similarly to mouse models, the lack of functional PLVAP in humans leads to severely compromised endothelial barrier function, resulting in edema, hypoproteinemia, hypoalbuminemia, hypertriglyceridemia and premature death. (Elkadri et al. 2015, Broekaert et al. 2018, Kurolap et al. 2018) The symptoms in humans verify the validity of the mouse models and underline the importance of PLVAP in human endothelial permeability and intestinal and vascular integrity.

2.1.1.5 PLVAP is a potential target for drug development and treatment

Loss of PLVAP is difficult to treat, but many pathological conditions have been associated with altered endothelial permeability and increased PLVAP expression. Cancer growth demands increased supply of blood and nutrients and therefore angiogenesis is often promoted by the tumor produced VEGF, which also induces PLVAP expression. Many anti-cancer treatments focus on targeting the tumor angiogenesis. Vascular permeability also increases in inflammation, because of the increased signaling and immune cell traffic in the inflamed tissue. The expression of PLVAP is reported to be increased in many pathological conditions such as nephritis in rats and the vasculature of many human tumors. (Strickland et al. 2005, Ichimura et al. 2008) In addition to cancer biology, PLVAP has been extensively studied in the field of eye and brain research. The blood-brain-barrier and blood-retinal-barrier are easily affected by changes in PLVAP expression and subsequent changes in permeability. (Bosma et al. 2018)

Inhibiting PLVAP expression has led to positive results in *in vitro* and *in vivo* experiments. The differential expression in healthy and cancer-associated endothelium was utilized by Wang et al. who successfully induced necrosis in

hepatocellular carcinoma by treating the tumor with anti-PLVAP antibody. (Wang et al. 2014) Suitably administered PLVAP inhibitors might also become a novel target for diabetic macular edema therapy and other retinal diseases. (Wisniewska-Kruk et al. 2016, Nakagami et al. 2019) Recently, PLVAP was successfully used as a target for drug delivery in the lungs. (Shuvaev et al. 2018) Because PLVAP is so widely expressed in the body, there is a heightened risk for adverse effects if the target tissue or cell type is not limited by selective formulation or administration route. Further research in mouse models and humans is needed but these results show that the function of PLVAP can be modified with encouraging outcomes.

2.1.2 The peripheral lymph node

Lymph nodes (**Figure 2**) are strategically located encapsulated secondary lymphoid organs which are connected to each other via the lymphatic vasculature and seeded by blood vessels. Lymph nodes develop during E15-E20.5 in mouse from lymphoid tissue inducer cells, which migrate from blood vessels in the prospective location of the lymph nodes. (Bovay et al. 2018) Hundreds of lymph nodes in the body serve as centrals for stromal components, APCs and circulating lymphocytes. The close interaction between the immune cells and the exposure to antigens and pathogens promote effective initiation of immune responses. Lymph nodes serve as important gatekeepers to prevent the spread of pathogens and metastatic cancer cells. (Brown et al. 2018) Therefore the traffic to and from the lymph node is carefully regulated.

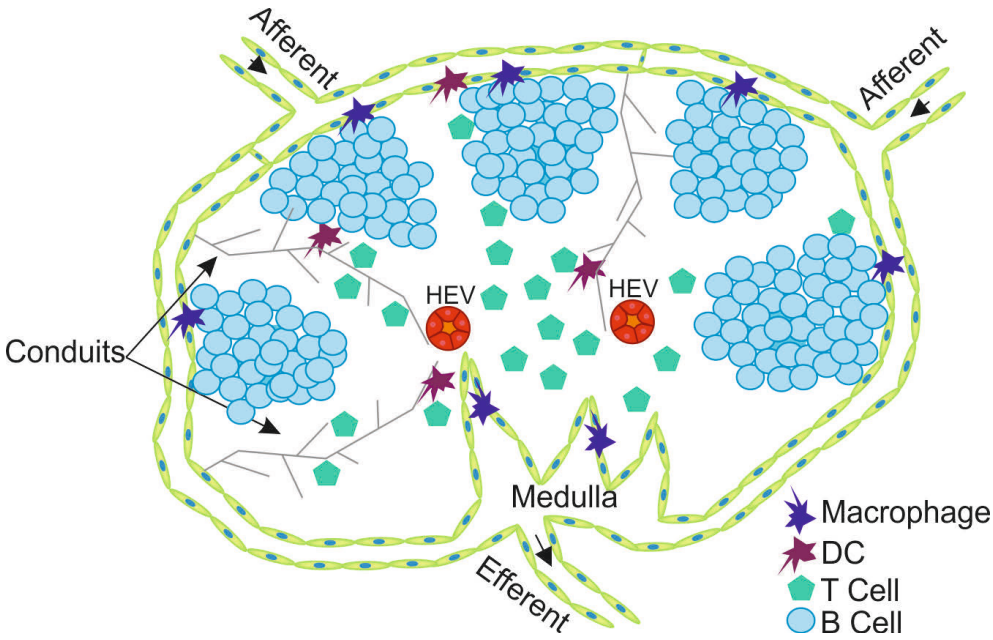

Figure 2. Schematic view of a peripheral lymph node. The arrowheads represent the direction of the lymph flow. HEV= High endothelial venule.

2.1.2.1 Subcapsular sinus

The lymph node is encapsulated by a bilayer of Prox1^{+} LECs which form a sinus that can be divided to a ceiling and a floor. LECs in the mouse subcapcular sinus (SCS) can be identified by their expression of markers such as Lyve-1, Prox-1 and podoplanin. (Baluk, McDonald 2008) The sinus is flushed by the constant flow of lymph, arriving via afferent lymphatic vessels from the periphery. The lymph is enriched with antigens and antigen presenting DCs, which together indicate the immune status of the tissues that drain to the specific lymph node. From the sinus, free flowing antigens can enter the parenchyma of the lymph node, but instead of pouring through the LEC floor, the entry of unbound antigens is size selective. Molecules of the molecular weight of 70 kDa or smaller have been shown to enter the lymph node. Exclusion of larger molecules could protect the lymph node lymphocytes from substances that might disturb the immune reaction, such as microbes and their soluble products. However, even if the lymph node is infected with a virus and enlarged in size, the filtering function of the LECs in the SCS floor remains unperturbed. (Gretz et al. 2000) Larger antigens need to be transported to the lymph node by migrating DCs. (Berney et al. 1999) The LECs in the SCS floor and medullary sinus are accompanied by CD169^{+} macrophages. They sample antigens from the lymph and either eliminate the antigens by phagocytosis, thus keeping the infections from spreading, or efficiently present the

antigens to the underlying B cells, subsequently activating the B cells. (Junt et al. 2007) The SCS continues all the way to the LEC lined medullary sinus of the lymph node and to the adjacent efferent lymphatic vessels. The medulla is phenotypically distinct from other parts of the LEC lined sinus, but its detailed function is not fully understood. (M'Rini et al. 2003, Iftakhar-E-Khuda et al. 2016) The efferent lymphatics facilitate the exit of lymphocytes through a process which is regulated by specific mechanisms involving sphingosine 1-phosphate and its receptors. (Allende et al. 2004)

2.1.2.2 Parenchyma

In addition to BECs and LECs, the lymph node parenchyma is inhabited by heterogeneous and functionally versatile stromal cells. Stromal fibroblastic reticular cells (FRCs) and follicular dendritic cells offer structural support and assist the homeostasis of the compartmentalized structure of the lymph node parenchyma. B cells gather together to form follicles directly under the SCS, but T cells reside more dispersed in the paracortex. Parenchymal cells define the limits of the lymphocyte compartments by controlling B and T cell CCR7-dependent movement within the parenchyma. (Bajénoff et al. 2006, Carrasco, Batista 2007) Even though the stromal cells and resident lymphocytes actively interact with each other, the development of the majority of stromal cells seems to be independent of B and T cells. (Fletcher et al. 2011) In addition to structural support, the stromal cells also express lymphocyte survival factors and take part in antigen presentation and induce self-tolerance among T cells. (Link et al. 2007, Cohen et al. 2010)

2.1.2.3 Conduit system

Immediately under the SCS begins the reticular conduit system, a three dimensional network of fibroblast reticular cell wrapped collagen strands and fibrils. This complex system travels from the SCS to the high endothelial venules (HEVs). The network is most dense in the T cell areas, but conduits can be found in the B cell areas as well. (Roozendaal et al. 2009) Resident DCs are in contact with the conduit system, probing the antigens travelling through the conduits. Immigrated DCs however do not seem to make contact with the conduit system. (Sixt et al. 2005) The conduit system is specialized in transporting several different kinds of cargo. Small antigens, larger IgM produced by resident B cells and even viral particles are able to enter the conduit system and be transported to the resident immune cells. (Thierry et al. 2018, Reynoso et al. 2019) The conduit system offers a pathway for antigens in the lymph to be quickly transported from the SCS to the HEV and be sampled on the way. Antigen transport via conduits happens in

minutes, in comparison to the hours it takes for an antigen transporting DC to arrive from the periphery. (Salomon et al. 1998, Gretz et al. 2000) Rapid import of antigens and immunoglobulins within the lymph node and further exit of antigen specific lymphocytes ensure an efficient response in peripheral tissues.

2.1.2.4 Resident leukocytes

Migration of lymphocytes into the lymph node is a complicated process. In brief, Naïve IgM^+ B cells and naïve $CD44^{Low}CD62L^{Hi}$ T cells travel to the lymph node parenchyma from the bone marrow and thymus respectively. (Gorelik et al 2003, Mempel et al. 2004) Both enter the PLN via the parenchymal blood vessels, HEVs, in a multistep process which is regulated by several factors, such as CCR7 in the lymphocytes, the HEVs and HEV associated DCs. (Young 1999, Stein et al. 2003, Girard et al. 2012, Veerman et al. 2019) T cells from the periphery arrive to the lymph node via the SCS, also this step seems to require assistance from the DCs in the lymph. (Braun et al. 2011) It is not clear which specific mechanisms are used during the migration of lymph-borne cells through the SCS LEC layer, but in general the migration across endothelium can occur between endothelial cells (paracellular route) or through an endothelial cell (transcellular route). Both migration routes involve distinct tethering of the LEC-layer by the lymphocytes, however the compared to paracellular migration, transcellular migration is more dependent on podosome function. (Carman et al. 2007) Expression of molecules such as CD73 on lymphocytes and Stabilin-1/CLEVER-1 and ICAM-1 on the ECs are some of the many molecules that have been shown to be important for migration of lymphocytes across the lymphatic and blood endothelium. (Xu et al. 2001, Ålgars et al. 2011, Shetty et al. 2011)

The purpose of the lymph node and its compartments is to provide effective communication between the immune cells and APCs, which is essential for the lymphocyte activation. Local APCs work with migrated DCs to present processed parts of encountered antigens to resident lymphocytes. (Allan et al. 2006) Priming by DCs induces activation markers such as CD25 and CD44, cytokine secretion and proliferation in T cells. T cell interaction is mostly contact-dependent, in comparison to the secretory manner in which B cells perform their functions. (Mempel et al. 2004) B cell activation occurs within the B cell follicle after exposure to the antigen with the help of their B cell receptor or a follicular DC or after interaction with an activated T cell. Activated $GL7^+$ B cells in the germinal center begin the process of differentiating into plasma cells and long lasting memory cells. (Gorelik et al. 2003, McHeyzer-Williams, McHeyzer-Williams 2005)

2.2 Macrophages

Macrophages are vital participants in several functions within the body, ranging from embryonic development to coordinating immune responses and maintaining homeostasis. Macrophages are specialized myeloid cells which take part in phagocytosis, scavenging, antigen presentation and cytokine production. Practically every tissue and organ is colonized with macrophages in the steady state and the macrophage counts usually increase in inflamed conditions. (Massberg et al. 2007, Ginhoux et al. 2016) Macrophages fight infections using several tactics. The initial, innate response sends macrophages among the first immune cells to the site of infection or trauma. Initial inflammation, caused in part by the macrophage produced cytokines and other cells that are attracted to the site by macrophage-derived chemokines, is often beneficial for the host and leads to healing. Macrophages promote resolving of the inflammation by inducing apoptosis in neutrophils and participating in clearing of the apoptotic cells. Excessive inflammation can lead to collateral damage in the surrounding tissue, which is why the macrophage response needs to be strictly controlled. (Koh, DiPietro 2011) In steady state, the macrophages patrol the tissues and phagocytose pathogens, bacteria and dead cells. Phagocytosed antigens are digested and the resulting peptides are presented to lymphocytes using the major histocompatibility complex (MHC) class I and II. Defects in the MHCII (in humans, human leukocyte antigen HLA) function can lead to disturbed immune responses, which are the cause for many chronic inflammatory and autoimmune diseases. (Menconi et al. 2008, Forabosco et al. 2009, Cho et al. 2014)

Macrophages are classified by many attributes, but many of the classifications are rather black and white and include various exceptions. Macrophages are notoriously plastic (Sica, Mantovani 2012), so the division that is fitting for the cell in one moment might not be accurate by the next. However, in many cases the classifications are useful and benefit scientific discussion, once an agreement has been reached about the characteristics. One just needs to keep in mind that the comparison of two types of macrophages usually leaves many in between.

A popular classification of macrophages is based on their activation by T cell products in *in vitro* settings. Classically activated M1 macrophages produce cytokines like TNFα in response to stimulation with IFNγ and LPS. Alternatively activated M2 macrophages are a large group of macrophages which are typically activated by cytokines such as IL-4 and IL-13. M1 macrophages are considered inflammatory, whereas the M2 macrophages are generally considered anti-inflammatory. (Sindrilaru et al. 2011) However, the growing knowledge concerning the heterogeneity of macrophages has raised questions about the broadness of the bipolar M1/M2 division. New classifications have been suggested to be based on for example the homeostatic activities of the macrophages. (Mosser,

Edwards 2008, Martinez, Gordon 2014) One of the newer ways of defining macrophage populations is to inspect their developmental origins.

Traditionally, macrophages were thought to arise from blood monocytes that patrol the body in the blood stream and enter a tissue in order to perform tissue surveillance and inflammation control. Mouse macrophages are commonly recognized by their "standard" surface markers F4/80, CD64, and MerTK, in addition to their ability to phagocytose. (Chakarov et al. 2019, Morris et al. 1991) More powerful analysis methods have revealed detailed differences in macrophage marker expression, which have been utilized to recognize macrophage subsets. In the recent decades, new insights to macrophage biology have come to light. Fate mapping studies have revealed that a proportion of macrophages in almost all adult tissues originate from the fetal era. These macrophages are not produced by the adult HSCs like typical adult macrophages, but self-renew within the tissue by downregulating transcription factors MafB and cMaf, and simultaneously activating a self-renewal gene network that is controlled by cell type–specific enhancers. (Soucie et al. 2016) Both the monocyte-derived and the tissue-resident macrophages have specific functions and both are necessary for the well-functioning immunity and homeostasis. The next sections discuss the origins of monocyte and fetal derived macrophages and their functions (see also **Box 1**).

Box 1. What is a monocyte or a macrophage?

The division between monocytes and macrophages has proved to be more complex than previously thought. Monocytes have been depicted as macrophage precursors, which are produced in the bone marrow and released into the blood stream where they circulate until they enter tissues and differentiate into macrophages or dendritic cells. However, it has become evident that monocytes and macrophages are not that easily categorized. Not all monocytes enter tissues and if they do, monocytes can exist in tissues without further differentiation to macrophages or dendritic cells. Macrophages on the other hand can develop and proliferate without monocyte-intermediates. The phenotype of either cell is markedly affected by tissue intrinsic cues and the age and pathological state of the subject, which complicates the classification of a "typical" monocyte or macrophage based on surface markers, origin or function.

Monocyte	**Macrophage**
Historically	Historically
• Produced in the bone marrow, circulates in blood and migrates to tissues, differentiates into macrophages and DCs	• Monocyte-derived tissue phagocyte
Today	Today
• Fetal liver / bone marrow origin • Inflammatory monocyte • Blood resident monocyte • Tissue monocyte with distinct localization • Endothelium support and antigen presetation	• Originates from yolk sac/fetal liver/ bone marrow • Interstitial or perivascular • Inflammatory and homeostatic functions
Typical markers	Typical markers
• $Ly6C^{-/+}$, $CD11b^{+}$, $CCR2^{+}$	• $CD11b^{+}$, $F4/80^{+}$, $MerTK^{+}$, $CD64^{+}$, $CD169^{+}$, $CD206^{+}$

2.2.1 Monocyte-derived macrophages

In adults, the bone marrow is the major hematopoietic organ. There, the transcription factor Myb –dependent hematopoietic stem cells (HSCs) produce different classes of hematopoietic progenitors, which in turn can produce mature cells of one or more lineages. (Wolber et al. 2002, Schulz et al. 2012) Monocytes and subsequent macrophages derive from a common myeloid precursor (MP), which is capable of generating macrophage and DC precursors (MDPs). MDPs, which express the chemokine receptor CX3CR1, can give rise to common DC precursors and monocytes. (Liu et al. 2009) Monocytes are short lived white blood cells, with the half-life of one to two days. In mouse, they are often divided by their expression of Ly6C to the $Ly6C^{High/+}$ monocytes and their progeny, the $Ly6C^{-}$ monocytes. The short life span means that monocytes need to be constantly produced and released to the blood stream, in order to meet the need for active patrolling. Monocytes circulate in the blood stream and increase migration to tissues once signs of distress are detected. (Yona et al. 2013) Monocytes that have entered the tissue begin to differentiate into macrophages and dendritic cells and combat threats like inflammation. (Massberg et al. 2007) In the steady state, many tissues, such as the intestines, rely on monocyte infiltration to maintain their tissue macrophage pool. (Bain et al. 2014)

2.2.1.1 Molecules that affect monocyte migration

In addition to the sufficient production of monocytes and monocyte-derived macrophages, their number in the circulation and tissues depends on the migration from the bone marrow to the blood vessels and from the vessels to the tissues. Monocyte traffic is crucial for the resolution of inflammation and therefore their migration has been the subject of several studies. Molecules such as CCR2 and Nur77 are important for the survival and correct migration of the $Ly6C^{+}$ and $Ly6C^{-}$ monocytes and the knock out mouse models of the two receptors are often used to study the monocyte function. The inherent problem of monocyte migration studies is the fact that the assays are often conducted under inflammatory conditions to promote the quantities of migrating cells. However, it is not always clear how well the information from inflammatory settings correspond to steady state.

The chemokine receptor CCR2 is expressed on a subset of monocytes. Its main ligand CCL2 is a major chemoattractant to $Ly6C^{High}$ monocytes. The production of monocytes in the bone marrow is CCR2 independent, but monocytes need CCR2 to exit the bone marrow into the blood circulation. CCR2 is also crucial for the tissue entry of monocytes. (Serbina, Pamer 2006, Tsou et al. 2007, Bonapace et al. 2014) Deletion of CCR2 inhibits monocyte entry to tissues, which has been shown to be

beneficial in protecting the host by curbing excess inflammatory response in pathological conditions. (Boring et al. 1998)

Nur77 (NR4A1) is one of the three members of the NR4A subfamily of orphan receptors in the steroid thyroid receptor family. (Martínez-González, Badimon 2005) Nur77 and the other members of the receptor family seem to have overlapping functions in the myeloid system, but the deletion of Nur77 alone has significant effects on the bone marrow differentiation and survival of $Ly6C^-$ monocytes. Loss of Nur77 leads to apoptosis of the $Ly6C^-$ monocytes in the bone marrow, but had no significant effect on the $Ly6C^+$ monocytes, which have low expression of Nur77. (Lee et al. 1995, Mullican et al. 2007, Nackiewicz et al. 2011)

2.2.2 Tissue-resident macrophages

Many tissues seem to harbor macrophages that differ from monocyte-derived macrophages in terms of their proliferation and persistence after inflammation has subsided. (Davies et al. 2011) Among others, the brain microglia (Ajami et al. 2007), liver Kupffer cells (Gomez Perdiguero et al. 2015) and alveolar macrophages in the lung (Guilliams et al. 2013) are all established before birth and show distinctive functions within their respective tissues. The composition of tissue macrophages seems to vary from tissue to another as some tissue-resident macrophages, such as macrophages of the heart, originate from several sources. Dick et al. revealed that the heart myocardium harbors distinct subsets of macrophages, some of which are maintained independently of blood monocytes and some partially or fully replaced by blood monocytes. (Dick et al. 2019)

2.2.2.1 The development of tissue-resident macrophages

The emergence of tissue-resident macrophages occurs in sequential waves during embryonic development (**Figure 3.**) and has been studied *in vivo* with mice in detailed fate-mapping studies. The first erythro-myeloid progenitors (EMPs) can be found in the extraembryonic yolk sac (YS) at embryonic day 7.0 (E7.0). (Palis et al. 1999) The CSF1R-dependent EMPs differentiate to macrophages in the YS. After the establishment of blood circulation at E8.5 the EMPs and the macrophages migrate from the YS to all tissues, including the brain and the liver before E10.5. EMPs in the fetal liver generate myeloid precursors, which give rise to fetal liver fetal erythrocytes, macrophages, granulocytes and monocytes until at least E16.5. (Gomez Perdiguero et al. 2015) At E10.5 the fetal liver is also seeded by HSCs from the aorta-gonad-mesonephros (AGM). HSCs in the developing embryo participate in the production of macrophages via monocyte intermediates and from this point onwards, the main hematopoiesis shifts from the YS to the fetal liver.

From the fetal liver, the monocytes enter the blood stream and seed all tissues at E12.5, excluding the brain which has been closed off by the blood-brain-barrier. (Ginhoux, Guilliams 2016) The brain is the only tissue, where the macrophage population, also known as microglia, exclusively originates from the YS. (Ginhoux et al. 2010) In other tissues, the YS-derived macrophages are at least partially superseded by the fetal liver -derived macrophages by E16.5 (and supplemented with bone marrow monocyte-derived macrophages postnatally). Finally, the macrophage production shifts from the liver to the bone marrow during E16 to 4 days postpartum. (Wolber et al. 2002) Around the time of birth and thereafter the HSC-derived monocytes in the bone marrow can participate to tissue-resident macrophage production as is the case with arterial macrophages. (Ensan et al. 2015) From this point onwards, the tissue-resident macrophages proliferate locally, but the postnatal systemic distribution of macrophages depends on the HSCs, the precursor cells and the rate of monocyte migration to the tissues. (Soucie et al. 2016)

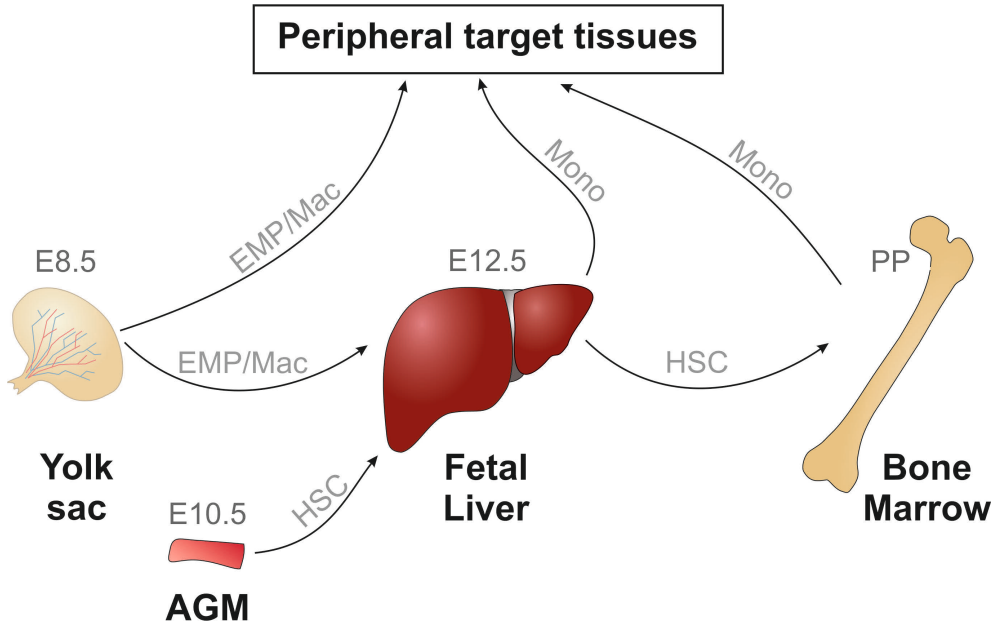

Figure 3. Macrophages in adult tissues originate from different developmental origins. Tissue-resident macrophages develop throughout embryonic development. In adulthood, macrophages replenishment relies on self-renewal of tissue macrophages, bone marrow HCSs and bone marrow -derived monocytes. AGM= aorta-gonad-mesonephros, EMP= erythro-myeloid progenitor, HCS= hematopoietic stem cell, Mac= macrophage, Mono= monocyte, PP= postpartum.view of a peripheral lymph node. The arrowheads represent the direction of the lymph flow. HEV= High endothelial venule.

Thus, in a simplistic view, tissue-resident macrophages originate mainly from the YS or fetal liver. In the adulthood, the two embryonic macrophage populations begin to resemble each other in their surface marker expression pattern. This makes it difficult to study the exact phenotypes and functions of the two embryonic populations in adult tissues. To some extent, cells of a distinct origin can be recognized with the help of inducible reporter mice. A conditional Cre-recombinase enzyme can be activated with tamoxifen to allow the transcription of a fluorescent protein in cells with a desired promoter, such as CX3CR1 or CSF1R. Timed tamoxifen pulsation will result in fluorescent cells of interest which can be followed until the life span of the cells or their progeny come to an end. (Yona et al. 2013)

The differences in macrophage characteristics between tissue-resident and monocyte-derived macrophages are partly explained by their developmental origin, but it has been suggested that the tissue environment could have an even greater effect on the macrophage phenotype. (Gautier et al. 2012, Lavin et al. 2014) Macrophages colonize the tissues early in the development, so it is reasonable that the populations are sculpted in part by the interactions with the tissue environment. Interestingly, the epigenetic programming by the tissue is able to mold the tissue-resident macrophages even from another tissue to resemble the organic population. (Lavin et al. 2014) Van de Laar however showed that the tissue-resident alveolar macrophages cannot be replaced by tissue-resident macrophages from any other tissue, but they can be replenished by adult donor alveolar macrophages or macrophage precursors from the YS or fetal liver (but not from the adult bone marrow). (Van de Laar et al. 2016) In myocardium, a small proportion of monocyte-derived macrophages is shown to be able to assume a nearly identical transcriptional profile to the resident macrophage populations. However, they are not able to redeem the spatially localized cardioprotective functions of the tissue-resident macrophages. (Dick et al. 2019)

These results indicate that the embryonic macrophages retain some level of plasticity throughout their life span. The unique profiles of the tissue-resident macrophages are affected by the tissue environment, but it seems unlikely that the tissue signals alone are capable of sculpting cells into functioning tissue-resident macrophages. Further research is needed to fully elucidate the contribution of the tissue environment and inherent genetic properties to the development of tissue-resident macrophages.

2.2.2.2 Tissue-resident macrophage functions

In addition to the task of fighting inflammation and infection when needed, tissue-resident macrophages take part in other functions. It is not surprising that the

tissue-resident macrophages, which are established during embryonic development, have a role in the development of organs. In the developing brain, microglia sculpts synaptic network and promotes synaptic maturation. (Paolicelli et al. 2011) During adulthood, the tissue-resident macrophages have many homeostasis maintaining functions, unique for their anatomical location. Peritoneal macrophages interact with peritoneal B1 cells and control the IgA production in the gut. (Okabe, Medzhitov 2014) The recycling of heme takes place in the spleen and the resident red pulp macrophages maintain iron homeostasis by phagocytosing red blood cells. (Haldar et al. 2014) Finally, the resident macrophages of the lung, alveolar macrophages participate in surfactant homeostasis. (Jubinville et al. 2019) These examples highlight the diverse tissue-specific functions that tissue-resident macrophages perform.

2.3 Mammary gland development and function

The mammary gland is a complex tissue that consists of adipocytes, mammary stem cells, epithelial cells, mesenchymal cells, vascular endothelial cells and immune cells. (Oakes et al. 2006, Inman et al. 2015) One of the most important functions of the mammary gland is to produce and distribute milk for offspring after pregnancy. To serve this purpose, the mature mammary gland stroma is a network of ducts, which are essentially hollow tubes lined with contractile epithelium. The ducts traverse the mammary gland and terminate in milk secreting alveoli (**Figure 4.**). The stroma between the ducts is comprised mainly of white adipocytes and fibroblasts. (Hennighausen and Robinson 2001, Gouon-Evans and Pollard 2002)

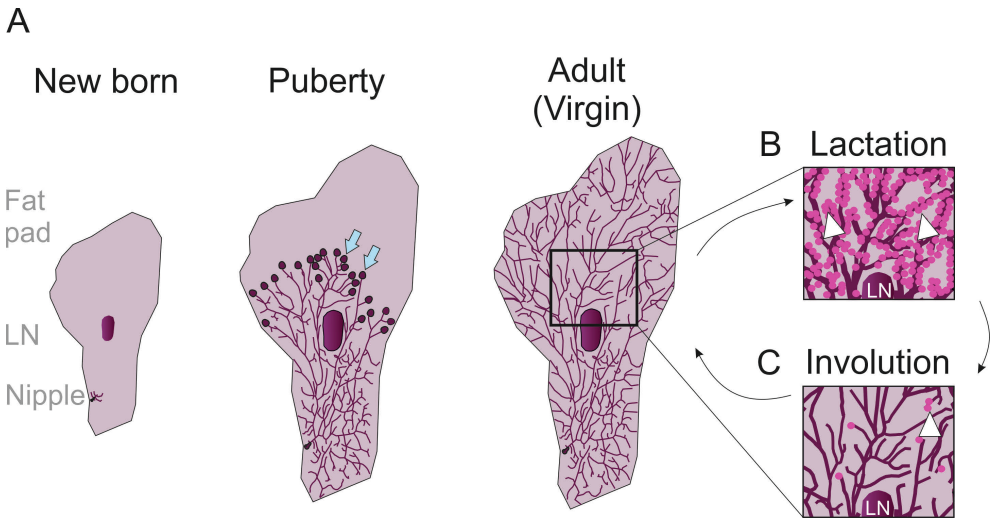

Figure 4. A schematic representation of mammary gland ductal development in different developmental stages (A). The blue arrows point to terminal end buds. The inserts show the budding alveolar structures (white arrow heads) during lactation (B) and the disappearance of the alveoli during involution (C). LN= lymph node.

The majority of the information on breast development and function originates from studies on mice. Clear differences between mouse and man include the number of mammary gland pairs per individual (one pair in humans compared to the five pairs in mice) and the extent of male mammary development. Unlike in mice, the human male breasts grow similarly to female breasts until puberty, until male hormones determine the smaller size of the breast. (Howard and Gusterson 2000) Additionally there is variation in the stromal architecture and the number of main ducts. Nonetheless, the developmental processes in the two species are similar and the mouse studies provide important insight to mammary gland cell interactions, signaling and function. (Parmar and Cunha 2004, Inman et al. 2015)

2.3.1 Prenatal development

The mammary gland development starts around halfway of pregnancy at E10 in mouse, in both sexes and is sparked by tissue interaction between epithelium and mesenchyme. Early breast development is largely regulated with local signaling and cell contact within the mammary tissue. Milk lines running along the ventral sides of the torso break apart to form placodes at E11, in response to avid signaling from the likes of *Wnt* ligands, *Tbx3*, *Fgf10* from the surrounding epithelium and mesenchyme. (Chu et al. 2004, Jerome-Majewska et al. 2005, Veltmaat et al. 2006, Inman et al. 2015) The placode forms into an epithelium ball that grows in size, changes shape and dives deeper into the underlying mesenchyme by E14. In male

embryos, testosterone signaling leads to degeneration of the bud, halting the formation of ducts. (Watson, Khaled 2008) In females, at E16-E18 the epithelial bud's neck elongates through the mesenchymal cells into the underlying cluster of preadipocytes. After the sprouts start initial branching into the future fat pad, they open up to create the lumen and nipple sheath, which are necessary for milk transport and nursing. (Hogg et al. 1983) Signaling from the fat pad guides the growth of the sprouts into 10-15 initial ductal branches, there after the ductal development slows down until puberty. (Couldrey et al. 2002)

2.3.2 Postnatal development

Postnatally, the mammary gland fat pad continues to grow in size in proportion to the body. In comparison to the prenatal regulation, postnatal development of the mammary gland ductal system is in larger part orchestrated by systemic hormones like estrogen and progesterone which are regulated by the pituitary/hypothalamus axis and ovary functions. (Hennighausen and Robinson 2001) In addition, the mammary environment and resident cells and their signaling continue to shape the developing ductal meshwork. (Gouon-Evans et al. 2000, Fata et al. 2000) During the first weeks of postnatal life, the existing rudimentary mammary ducts grow slowly. (Watson, Khaled 2008) Once puberty begins, the growth escalates and the avidly proliferating terminal end buds (TEBs) at the end of the ducts further invade the fat pad, sprouting onwards and laterally, finally filling 60% of the breast with the ductal tree. (Macias, Hinck 2012) The ductal branches grow until they have reached the fat pad border at around 10-12wks of age. (Watson, Khaled 2008) At this point the mammary gland has reached maturity. The size of the ductal network in virgin mice fluctuates in response to the cyclical hormonal cues during the estrous cycle (menstruation in humans). During pregnancy and lactation, the ductal epithelium forms milk-secreting alveoli which disappear during the involution phase after weaning. (Förster et al. 2002, Oakes et al. 2006, Joshi et al. 2010)

2.3.3 Macrophages in the mammary gland

Macrophages are a part of normal mammary gland stroma and have been detected in the mammary gland from 2wk onwards. Macrophages promote the ductal growth by clearing the apoptotic epithelial cells in the TEB, stabilizing the morphology of the TEB and by rearranging the collagen fibers around the growing TEB. Macrophages localize to the neck of the TEB and also close to the nipple. (Gouon-Evans et al. 2000, Ingman et al. 2006) The macrophage localization in the vicinity of ducts is often attributed to the local chemokines produced by the epithelium, such as the CSF1, a major growth factor and chemoattractant for

macrophages. (Ryan 2001) CSF1-deficient mice experience reductions in macrophage number in the mammary gland. (Gouon-Evans et al. 2000) CSF1 levels and the macrophage numbers in the wild type (WT) adult mouse fluctuate during the different phases of the estrus cycle, however their peaks do not correlate with each other. (Dasari et al. 2014, Chua et al. 2010) Van Nguyen et al. showed that the CSF1 does recruit macrophages, but is not necessary for their recruitment, suggesting that other signals are also responsible for the macrophage presence in the mammary gland. (Van Nguyen, Pollard 2002) Ductal epithelium is produced by the mammary stem cells and mammary macrophages have been shown to be important for the mammary gland stem cell activity and epithelial proliferation. (Gyorki et al. 2009) Chakrabarti et al. showed that the close proximity between macrophages and mammary gland stem cells can be explained by the contact-dependent Notch/Wnt signaling loop between the stem cells and macrophages. This signaling not only promotes stem cell activity but also maintains the macrophage number and activity in the mammary gland. (Chakrabarti et al. 2018)

2.3.3.1 Mammary macrophages as therapeutic targets

Breast cancer is the most prevalent cancer among women world-wide and in various countries the incidence rates have been rising. Even though there are several treatment strategies to tackle breast cancer, it still is the leading cause of cancer death. (Bray et al. 2018) In addition to traditional therapy options like hormone blocking strategies, surgery, chemotherapy and radiation, new unconventional approaches are needed. All the available treatments have undesired adverse effects and even though they are effective in many cases, there are still many patients who receive little to no benefit from the current methods. Immunomodulatory therapies are based on the concept that the patient's resident immune cells can be modulated to recognize and fight off the cancerous cells. (Umansky, Sevko 2012) Coissieux et al. used mice and approached the problem by curbing the recruitment of $CCR2^{+}$ monocytes to the mammary tumor by inhibition of CCL2 signaling. This led to reduction in macrophages numbers in the primary breast cancer tumor, but the cessation of inhibition surprisingly led to increased metastasis and accelerated death. Cessation of treatment resulted in the macrophage-mediated production of VEGF-A in the metastatic tissue resulting in increased angiogenesis in the metastatic tumors. (Bonapace et al. 2014) Thus, immunotherapies need to be carefully examined and permanent reprogramming of the tumor microenvironment or complete elimination of tumors is necessary to avoid a risk of negative rebound.

So far, there have been some successful attempts in the immunomodulatory approaches. Hu et al. showed *in vitro* that high-intensity focused ultrasound

treatment of breast cancer tumors led to tumor cell -derived danger signals, which activated macrophages and enhanced anti-tumor immunity. (Hu et al. 2005) Blocking of the CSF1/CSF1R signaling in a mouse model of pancreatic ductal adenocarcinoma efficiently depleted tumor associated macrophages. The reduction of macrophages significantly enhanced the efficacy of immunotherapy which was based on the inhibition of T-cell checkpoint with antagonists for cytotoxic T lymphocyte antigen 4 (CTLA4) and Programmed cell death-1 (PD1). Combination of the two approaches resulted in the successful regression of tumors. (Zhu et al. 2014) Modulation of the existing immune cells requires a vast amount of detailed information on the target tissue stromal cells and the particular primary and metastatic cancer cells. It is imperative to understand the steady-state profile of the immune cells within the target tissue, before attempting to reinstate the homeostasis.

It is known that the mammary gland harbors macrophages at least throughout the postnatal development, but the phenotype of the macrophages is still only superficially researched and further investigation with current methods is needed. Macrophages are also found in breast cancer both within the tumor and in the tumor environment, where they have been extensively studied. However, macrophage presence in breast cancer has been linked to positive and negative outcomes. (Medrek et al. 2012) This could imply that the macrophages are not homogenous in phenotype and that the tumor environment might affect the different macrophages in different ways.

3 Aims

Correctly functioning endothelial cells and the vasculature they line are crucial for the well-functioning body. Endothelium is involved in embryonic develop-ment, maintenance of vessel integrity and cell traffic control. This thesis aims to investigate the role of endothelial cell permeability in cell and molecule ex-change in physiological state and to elucidate the consequences of perturbed endothelial cell function.

The specific aims of the study were:

1. To explore the role of PLVAP in the lymphatics, especially in the endothelial cells of the lymph node sinus.
2. To learn how the PLVAP expressing lymphatic endothelial cells affect the immune response within the lymph node.
3. To determine how the endothelial protein PLVAP affects migration of macrophage precursors during development.
4. To elucidate the macrophage status of virgin steady-state mammary gland.
5. To examine the functions of tissue-resident macrophages in the mammary gland.

4 Materials and Methods

4.1 Animals (I-III)

This thesis comprises the results from studies performed on several mouse strains. All mice were housed under controlled, specific pathogen free conditions and a 12 h light-dark cycle at a temperature of 22 °C in the animal facilities of University of Turku (Turku, Finland). The mice had *ad libitum* access to chow (irradiated standard pellets) and water. All animal experiments were reviewed and approved by the Ethical Committee for Animal Experimentation in Finland. Experiments were carried out in adherence with the rules and regulations of the Finnish Act on Animal Experimentation (497/2013) and in accordance to the 3R-principle under Animal license numbers: 3791/04.10.03/2011 and 5587/04.10.07/2014 and 6211/04.10.07/2017. Both genders were used in the experiments (except in mammary gland analyses) in Publications I and II. In III, only females were used unless indicated otherwise. Mice were used at indicated ages. When gene-modified mice were used, age-, sex- and strain-matched WTs or littermates were used as controls. For embryonic time points, the female was introduced to the male for one night and the morning of the vaginal plug detection was considered as embryonic day E0.5.

4.1.1 Wild type strains (I-III)

Commercially available WT mice C57BL/6J, C57BL/6N, NMRI, F1 hybrid C57BL/6;129 and BALB/c mice were from Charles River, The Jackson Laboratory or Janvier Labs.

4.1.2 Knock out strains (I-III)

Cav1tm1Mls/J (*Cav1*$^{-/-}$, stock004585), *Ccr2*$^{-/-}$ mice (stock 004999) and *Nur77*$^{-/-}$ mice (stock 006187) were purchased from The Jackson Laboratory. Nt5et^{m1Lft} (*Nt5e*$^{-/-}$) and Aoc^{3tm1Salm} (*Aoc3*$^{-/-}$) mice have been described in (Stolen et al. 2005) and (Thompson et al. 2004) respectively.

4.1.3 Generation of conditional *Plvap*$^{-/-}$ mice (I)

PLVAP-deficient mice were generated at Taconic Artemis and thereafter were bred in the University of Turku animal facilities. In brief, the *Plvap* targeting vector was constructed with bacterial artificial chromosome clones from the C57BL/6J RPCI-24 bacterial artificial chromosome clones library. Translation initiation site -containing exon 1 and approximately 1.5 kb of the sequence upstream of exon 1 (promoter region) was flanked by *lox*P-sites. The positive selection marker PuroR was inserted into intron 1. The linearized targeting vector was transfected into the Taconic Artemis Art B6/3.6 ES cell line on the C57BL/6NTac genetic background. The homologous recombinant clones (PuroR+) were isolated and confirmed by Southern blot analysis and correct homologous recombination at the 5′ side (5ext1 probe: sense, TGCTCTGGAAGCTGAGCTTGGCACC, and antisense, TGCTGCCACCTAGGCAAGTCTTGG) and 3′ side (3ext2 probe: sense, GGGTAGAGGTTCAGGTCTGTCCTC, and antisense, AGAGCTGAGTTGTGACCCAGTCACC). The proximal *lox*P-site was detected in all clones. The targeted embryonic stem cells were injected into BALB/c mouse blastocysts, and chimeric mice were generated. Highly chimeric male mice were bred to deletor (C57BL/6-Tg(CAG-Flpe)2Arte) female mice which expressed Flp recombinase in order to confirm the germline transmission and to generate mice with conditional deficiency in Plvap (*Plvap*$^{F/+}$). *Plvap*$^{+/-}$ mice were generated by breeding *Plvap*$^{F/+}$ mice to CAG-Cre (NMRI-Tg(CAG-Cre)) mice. Intercrossing of *Plvap*$^{+/-}$ mice resulted in *Plvap*$^{-/-}$ mice in addition to the WT littermate controls and *Plvap*$^{F/+}$ mice. *Plvap*$^{-/-}$ mice on a mixed background (including BALB/c, C57BL/6N and NMRI strains) were used in the experiments in Publications I-III.

4.1.4 Tamoxifen inducible models (II, III)

Inducible *Plvap*$^{F/F}$*; CAGGCre-ER*^{TM} mice were produced by crossing conditional *Plvap*$^{F/F}$ mice with a *CAGGCre-ER*^{TM} *(B6.Cg-Tg (CAG-cre/Esr1)*$^{5Amc/J}$ deletor Cre mouse line (stock 004682, The Jackson Laboratory). Conditional *Plvap*$^{F/F}$ mice were crossed with *Lyve-1-Cre* mice (*Lyve1*$^{tm1.1(EGFP/cre)Cys}$, (stock 012601, The Jackson Laboratory)) to generate *Plvap*$^{F/F}$*; Lyve-1-Cre* mice. (II)

For fate mapping studies, R26R-EYFP mice (stock 006148) were crossed with either *Cx3cR1*$^{Cre/ERT2}$ (stock 020940) or Csf1r-Mer-iCre-Mer (stock 019098), all purchased from The Jackson Laboratory. (III)

4.2 Macrophage depletion (I, II)

4.2.1 Depletion of existing macrophages (I)

WT mice were injected s.c with clodronate-loaded or control liposomes (ClodrodateLiposomes.com). Analysis of the macrophage depleted tissues or further experiments were performed seven days after clodronate treatment.

4.2.2 Depletion of YS-derived macrophages prenatally (II, III)

Pregnant (E6.5) WT female mice were treated with a single i.p injection of 3 mg of the CSF1R blocking antibody (clone AFS98, BE0213 Bio X Cell) or rat IgG2a control antibody (clone 2A3, BE0089 Bio X Cell) to deplete the YS-derived macrophages in the offspring.

4.2.3 Depletion of tissue-resident macrophages postnatally (III)

Tissue-resident macrophages were depleted by injecting 2 wk old WT mice cyclically with anti-CSF1 antibody and clodronate: three doses of CSF1 neutralizing antibody (Clone 5A1, BE0204 Bio X cell) or control IgG (clone HRPN, BE0088 Bio X cell) were given i.p, followed by three doses of clodronate or control liposomes i.v (Liposoma) on the following day. Mice were sacrificed at indicated time points after the last clodronate administration.

4.3 Colony forming assay (II)

Adult bone marrow from the femur and livers of E12.5 embryos were collected and seeded in 1 ml of M3434 Methocult medium (Stem Cell Technologies) into 35-mm culture dishes in duplicates. Amount of colonies were counted after 7 day culture at 37 °C with 5% CO2.

4.4 Immunoblot analysis (I)

Spleen stromal elements from mechanically dissociated spleens were lysed for 1 h. The sample was centrifuged and aliquots of the lysate were mixed with non-reduced Laemmli's sample buffer and resolved by 5–15% SDS-PAGE. PLVAP was using with MECA-32 after transfer to nitrocellulose and anti-GAPDH (5G4 mAb 6C5; Hytest) was used as a loading control. Enhanced chemiluminescence detection (ECL) system (Amersham) was used.

4.5 Interaction studies (II)

4.5.1 Generation of PLVAP-Fc fusion protein (II)

A fusion protein with the extracellular domain of mouse PLVAP fused to a human IgG2 Fc-tail was generated. The extracellular domain of mouse PLVAP (amino-acid residues 48–438) was PCR-cloned (Phusion High-Fidelity DNA Polymerase, ThermoFisher Scientific) from a full-length cDNA clone for mouse PLVAP (MR206983, Origene) using primers introducing EcoRV and NheI digestion sites. The amplified fragment was purified and annealed to EcoRV and NheI digested pFUSEN-hG2Fc vector (InvivoGen). Sequencing and immunoblotting were used to verify the integrity of the construct. The expression plasmid was transfected into HEK293-EBNA cells (CRL-10852, from ATCC) and the cells were cultured for 2-3 days. CD4–Fc-chimaera was used as a control in the interaction studies.

4.5.2 Heparin affinity pull down assay (II)

Heparin- or streptavidin-coupled agarose beads (Sigma or GE Healthcare, respectively) were washed, and blocked with TBS with 1% BSA. The beads were rocked with either total protein lysates from E14.5 WT livers or the PLVAP–Fc and CD4–Fc fusion proteins. After washing, the bead-bound molecules were eluted and subjected to SDS–PAGE separation.

4.5.3 Far western (II)

Recombinant mouse neuropilin-1 (R&D Systems, 5994-N1) and recombinant mouse $VEGF_{164}$ (R&D Systems, 493-MV) were spotted onto filters. The PLVAP–Fc fusion protein was allowed to bind to the immobilized proteins in the presence or absence of heparin (Leo Pharma) for 2 h. ECL and HRP-conjugated anti human IgG antibody was used to visualize the bound fusion protein.

4.5.4 Proximity ligation assay (II)

VEGF interaction with PLVAP in fetal livers was evaluated with a proximity ligation assay (PLA). Briefly, anti-VEGF (46154, Abcam) or control primary antibody were detected by Duolink *in situ* PLA probe anti-rabbit PLUS (DUO92002, Sigma). Anti-PLVAP antibody was directly conjugated to MINUS PLA probe with Duolink *in situ* probemaker MINUS kit (DUO92010, Sigma). Detection reagent red (DUO92008, Sigma) was used to detect the ligated probes after amplification. Anti-PLVAP was also detected with an additional secondary

antibody. Finally, the nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) and samples were mounted in Mowiol and imaged.

4.5.5 Co-immunoprecipitation assay (II)

Freshly isolated WT fetal livers were briefly lysed before pelleting. The supernatants were incubated with an anti-VEGFA antibody or a negative control for 5 h. Protein G beads were added for 1 h. The beads were washed and the bound proteins were eluted in non-reducing Laemmli's sample buffer. The proteins were separated in SDS–PAGE and immunoblotted for VEGF and PLVAP using IRDye-conjugated second-stage reagents and Odyssey imager.

4.6 Fluorescence-activated flow cytometry (I-III)

4.6.1 Isolation of the cells for single-cell suspension (I-III)

For flow cytometry analyses the excised tissue samples were processed into single-cell suspension. Briefly, most tissues were first mechanically dissociated, followed by a 0,5-2h enzymatic digestion in Hank's balanced-salt buffer containing collagenase D (1 mg/ml) and DNAse I (50 μ g/ml). Tissues were then filtered and washed before staining for flow cytometry. Some tissues needed a method with some exceptions: livers were dissociated using Gentle MACS C-tube (Miltenyi Biotech) and immune cells were purified via OptiPrep density gradient centrifugation (Sigma D1556). Brains were digested, followed by a Percoll gradient isolation of microglia before staining. Bone marrows were isolated by gently crushing and extensively filtering the femurs before staining. Lamina propria cells from the colon were isolated by an enzymatic digestion. Peritoneal cells were collected by gently rinsing the peritoneal cavity with medium containing heparin and serum. Erythrocytes in blood and spleen were lysed by hypotonic lysis. (I-III) In some experiments lymph nodes were gently mechanically teased to detach the lymphocytes without enzymatic digestion. For LEC analyses, minced lymph nodes were digested for 15 min at 37 °C with 25 μ g/ml liberase TM and 100 μ g/ml DNase I. Samples were delicately passed through Pasteur pipettes and filtered, the remaining undigested fragments were collected and subjected to four additional cycles of digestion with fresh enzymes. Cell suspensions from each cycle were pooled and stained. (I)

Total leukocyte numbers were determined by establishing the absolute numbers of viable cells in the cell suspension with a cell counter (Cellometer Auto 2000, Nexcelcom) and the percentage of $CD45^+$ cells with flow cytometry. Absolute

blood leukocyte numbers were determined with a hemocytometer (VetScan HM5, Abaxis). (II)

4.6.2 Staining of cells for flow cytometry (I-III)

Before staining the single cell suspensions, the Fc-receptors were blocked with purified anti-CD16/32 to reduce non-specific binding of the antibodies. Samples were then stained with either directly fluorochrome-conjugated antibodies or a primary antibody and a fluorochrome-conjugated secondary antibody. Antibodies used in the three publications are listed in **Table 1**. Finally, after washing (and when necessary, fixation of the samples) samples were analyzed and collected with cell sorters FACSAriaII (BD) (I-III) or Sony SH800Z (Sony Biotechnology) (II), or analyzed with LSRII (BD) (I) or LSR Fortessa (BD) (I-III). Results were analyzed and visualized using FACSDiva software (I) or FlowJo-software (Tree Star/ FlowJo LLC) (I-III).

Table 1. Antibodies used in the flow cytometric stainings.

Antibody	Company	Cat#	Pub.
B220-PE	BD Biosciences	553089	I, II
B220-BV421	BD Biosciences	562922	II
B220-Pacific Blue	BD Biosciences	558108	I, II
CD3-Pacific Blue	BD Biosciences	558214	I
CD4-APC	BD Biosciences	552051	I, II
CD4-APC-CY7	BD Biosciences	561091	I
CD4-APC-Cy7	BD Biosciences	552051	II
CD4-A647	BD Biosciences	557681	II
CD4-PE	BD Biosciences	553048	II
CD8-PerCP-Cy5,5	BD Biosciences	551162	I, II
CD8-PE	BD Biosciences	553032	II
CD8-BV650	BD Biosciences	563234	II
CD11b-PE	BD Biosciences	553311	I-III
CD11b-APC-Cy7	BD Biosciences	561039	I, II
CD11b-APC-Cy7	BD Biosciences	557647	III
CD11b-BB515	BD Biosciences	564454	II, III
CD11b-BV786	BD Biosciences	740861	III
CD11c-BV421	BD Biosciences	562782	I-III
CD11c-PerCP-Cy5,5	BD Biosciences	560584	I, II
CD16/CD32 (2.4G2)	Bio X Cell	CUSTOM24G2	III

Antibody	Company	Cat#	Pub.
CD25-APC	eBiosciences	17-0251-81	I
CD31-APC	BD Biosciences	551262	I
CD34-PerCP-Cy5,5	BD Biosciences	128607	II
CD41-PE	BD Biosciences	561850	II
CD41-FITC	BD Biosciences	553848	II
CD42.2 PerCP-Cy5,5	BD Biosciences	552950	I
CD44-PerCP-Cy5,5	eBiosciences	45-0441-80	I
CD45-APC-Cy7	BD Biosciences	557659	I, II
CD45-V500	BD Biosciences	561487	I, II
CD45-PerCP-Cy5.5	BD Biosciences	550994	II, III
CD62L-A647	BioLegend	104421	I
CD64-PE	BioLegend	139304	III
CD115-PE-Cy7	eBioscience	25-1152-80	II, III
CD117-BV510	Biolegend	135119	II
CD127 (IL7) -PeCy7	BD Biosciences	560733	II
CD135 (Flt-3)-BV421	BD Biosciences	562898	II
CD169-PE	BioLegend	142403	I
CD206-A488	Adb Serotec	MCA2235A488	II
CD206-BV650	BioLegend	141723	III
CD304-PE	eBiosciences	12-3041-82	II
F4/80-A647	BioLegend	122610	I
F4/80-PE	Abcam	Ab105156	II
F4/80-A488	eBioscience	53-4801-82	III
F4/80-A647	Bio-Rad/AbdSerotec	MCA497A647	III
CXCR5-APC	BD Biosciences	560615	I
GL7-A647	BD Biosciences	561529	I
IgD-A647	BioLegend	405708	I
IgM-APC-A780	eBiosciences	47-5790-80	I
Ly6C-BV421	BD Biosciences	562727	II, III
Ly6C-FITC	BD Biosciences	553104	II
Ly6G-BV510	BioLegend	127633	II, III
Lin-APC	BD Biosciences	558074	II
MerTK-PE	BioLegend	151505	III
MHCII-PE-Cy7	eBioscience	25-5321-82	I-III
Podoplanin-PE-Cy7	BioLegend	127412	I
S1P1	Santa Cruz	sc25489	I

Antibody	Company	Cat#	Pub.
Sca-1-PE	R&D Systems	FAB1226P	II
Siglec - F -PE -CF594	BD Biosciences	562757	II, III
VEGFR1-PE	R&D Systems	FAB4711P	II
VEGFR2-PE	eBiosciences	12-5821-81	II
Armenian hamster IgG1 (λ1) -PerCP-Cy5,5	BD Biosciences	560554	I, II
Armenian hamster IgG1 (λ2) -BV421	BD Biosciences	562919	I, II
Armenian hamster IgG-PerCP-Cy5,5	Biolegend	400931	II
Goat anti Rabbit A488	Life Technologies	A11008	I
Hamster IgG-PE-Cy7	BioLegend	402009	I
Lin-Isot conrol cocktail-APC	BD Biosciences	558074	II
Rat IgG1-APC	eBiosciences	17-4301-81	I
Rat IgG1-FITC	BD Biosciences	553924	II
Rat IgG2a-A488	Adb Serotec	MCA1212A488	II
Rat IgG2a-A647	BD Biosciences	557690	II
Rat IgG2a-A647	BioLegend	400526	I
Rat IgG2a-APC	BD Biosciences	553932	I
Rat IgG2a-APC–A780	eBiosciences	47-4321-82	I
Rat IgG2a-BV421	BD Biosciences	562602	II
Rat IgG2a-BV510	BD Biosciences	562952	II
Rat IgG2a-BV650	BD Biosciences	563236	II
Rat IgG2a-Pacific Blue	BD Biosciences	558109	I, II
Rat IgG2a-PE	BD Biosciences	553930	I
Rat IgG2a-PE-CF594	BD Biosciences	562302	II
Rat IgG2a-PE-Cy7	eBiosciences	25-4321-81	II
Rat IgG2a-PerCP-Cy5,5	BD Biosciences	550765	I, II
Rat IgG2b-APC-Cy7	BD Biosciences	552773	I, II
Rat IgG2b-A647	Biolegend	400626	II
Rat IgG2b-BB515	BD Biosciences	564421	II
Rat IgG2b-BV510	Biolegend	135119	II
Rat IgG2b-PE	BD Biosciences	553989	I
Rat IgG2b-PE-Cy7	eBiosciences	25-4031-81	I, II
Rat IgG2b-PerCP-Cy5,5	BD Biosciences	550764	II
Rat IgG2b-PerCP-Cy5,5	eBiosciences	45-4031-80	I
Rat IgG2b-V500	BD Biosciences	560784	I, II
Rat IgM-A647	BD Biosciences	560892	I
Rat IgM-FITC	BD Biosciences	553942	II

4.7 *In vivo* migration assays (I)

WT donor splenocytes were mechanically isolated and erythrocytes were lysed by hypotonic lysis. The cells were labeled with 0.5 μM CFSE (carboxyfluorescein diacetate succinimidyl ester) in RPMI-1640 medium, and washed twice after 15min incubation in +37C. The cells (10×10^6 cells in 20 μl PBS) were injected s.c into the hind paws of the recipients. Draining lymph nodes were processed for microscopy after 4 or 8 hour migration.
In certain experiments, the CFSE-labeled cells were injected in combination with a PE-conjugated or unconjugated anti-MECA-32 (1µg/mouse). PE-conjugated or unconjugated isotype-matched antibodies were used as controls. Lymph nodes were collected after 4 h and processed for microscopy. Alternatively, lymph nodes were collected after 8 h and analyzed by flow cytometry for quantification of immigrated CFSE+ cells and subpopulations.

4.8 *In vitro* transmigration assays (I)

For functional end-point transmigration experiments, confluent human lymph node LECs (SciencCell Research Laboratories) were activated for 19 h with human recombinant TNF (50 ng/ml; R&D Systems). Cultured, washed mononuclear cells from donors were applied to the top of LEC cultures in HBSS medium containing 1% human serum albumin, and were allowed to interact for 20 min at 37 °C in a humidified incubator. Thereafter the adherent cells were fixed with 4% PFA. The cells were permeabilized, stained and imaged.

4.9 *In vivo* uptake experiments (I, III)

The antigen up-take by resident macrophages and dendritic cells of the lymph node was studied using recombinant AOC3-A647 (180 kDa) and ovalbumin (OVA)-A488 (45 kDa). Mixture of both conjugated antigen (0,5 µg of each) was injected s.c to mice and the draining lymph node was collected 30 min later. The lymph node was processed for flow cytometry and the frequency of each cell type containing the fluorescent antigens was determined with flow cytometry. (I)

In other experiments, immune complexes were produced by incubating OVA-Atto488 (41235 Sigma, 2 mg/ml in PBS) at 5:1 molar ratio with rabbit polyclonal anti-OVA IgG for 1h at 4C. 100 μl of OVA-SIC, or PBS as a control, was injected i.v. Alternatively, 10 μg of fluorescently labeled acetylated low-density lipoprotein (LDL; Alexa Fluor 488 conjugated, L23380 Thermo Fisher Scientific) or 0.8 mg of 500 kDa Dextran (fluorescein, D7136 L23380 Thermo Fisher Scientific) were administered i.v. After 1 h (dextran) or 2 h (OVA-SIC and LDL) the mice were sacrificed and the mammary gland was collected and processed for

flow cytometry. The mean fluorescence intensity (MFI) of each label in the PBS-treated mice was subtracted from MFI for the corresponding label in the test compound treated mice to determine the specific MFI for each test compound. (III)

4.10 *In vivo* conduit-filling studies (I)

Recombinant human AOC3 (Peprotech), a mixture of fluorescently labeled dextrans of various molecular weights (all from Molecular Probes) or fluorescent 20 nm quantum dots (QTracker) were injected s.c into the hind paw. After one minute (AOC3), two minutes (dextrans) or 10 minutes (quantum dots), the mice were sacrificed and the draining lymph node was collected. The sample was fixed overnight in 1% PFA or for 2 h at room temperature and for 2 h at 4 °C in 4% PFA. Dextran and quantum dot samples were dehydrated overnight in 30% sucrose and mounted in Optimal Cutting Temperature compound (OCT). Sections were cut, stained and imaged. After fixation with PFA, the AOC3 was detected with a polyclonal anti-AOC3 (PAS-2542; Harlan Sera-Lab).

4.11 Proliferation assay (III)

Mice were injected i.p with 120 μl of 10 mg/ml solution of bromodeoxyuridine (BrdU, BD Bioscience) and sacrificed two hours later. Mammary glands were processed for flow cytometry, but after immunofluorescence staining, the cells were further stained with FITC–conjugated BrdU antibody (BrdU Flow Kit, BD Bioscience) and analyzed with flow cytometry.

4.12 Mass cytometry (III)

The cells were isolated in the same manner as for flow cytometry. The cells were stained for dead cells with Cell-ID Cisplatin (Fluidigm; cat. 201064). After washing and Fc-blocking, the cells were stained with heavy-metal isotope–labelled antibodies. Antibodies used for stainings are listed in **Table 2**. After washings, the cells were incubated with DNA intercalation solution (1:1000 Cell ID Intercalator-103Rh in MaxPar® Fix and Perm Buffer; cat. 201067; Fluidigm), fixed with 4% PFA overnight. The pelleted cells were resuspended to MaxPar Water (cat. 201069; Fluidigm) immediately prior to data acquisition with a Cytometry by time of flight (CyTOF) mass cytometer (Helios, Fluidigm). The T-distributed Stochastic Neighbor Embedding (t-SNE) algorithm was applied for visualization of the multidimensional data in two-dimensions (viSNE, Cytobank, https://www.cytobank.org). The distinct $CD45^+$ cell populations were manually assigned to different leukocyte subpopulations. To allow direct comparison with flow cytometric analyses in certain

experiments, the mass cytometry data was manually analyzed using bi-axial blotting. Alternatively, the data were uploaded to the VorteX clustering environment (https://github.com/nolanlab/vortex/releases/tag/29-Jun-2017). Unsupervised hierarchical clustering with X-shift algorithm was performed with 15 selected parameters on the datasets from 5wk and 3 month old mice. The default settings were used for the clustering, with nearest density estimation (K) from 150 to 10, with 30 steps. Then, all data sets were selected and the elbow point (K) was calculated. All 13 clusters were selected and the force-directed layout was created (ForceAtlas2 algorithm). The distinct $CD45^+$ cell clusters were manually assigned to different leukocyte subpopulations. The layout and the visualization were produced with Gephi 0.9.1 (https://gephi.org).

Table 2. Antibodies used in mass cytometry.

Antibody	Company	Cat#
B220-159Tb	Fluidigm	3159015C
CD4-172Yb	Fluidigm	3172003C
CD8a-168E	Fluidigm	3168003C
CD11b-148Nd	Fluidigm	3148003C
CD11c-142Nd	Fluidigm	3142003C
CD45-175Lu	Fluidigm	3175010C
CD64-151Eu	Fluidigm	3151012C
CD80-171Yb	Fluidigm	3171008C
CD117-173Yb	Fluidigm	3173004C
CD206-169Tm	Fluidigm	3169021C
CD274-153Eu	Fluidigm	3153016C
CX3CR1-164Dy	Fluidigm	3164023C
F4/80-146Nd	Fluidigm	3146008C
Ly6C-162Dy	Fluidigm	3162014C
Ly6G-141Pr	Fluidigm	3141008C
MerTK-PE	BioLegend	151505
MHCII-174Yb	Fluidigm	3174003C
Anti PE (PE004) 158HO	Fluidigm	3165015C
Siglec - 1 -170Er	Fluidigm	3170018C
Siglec - F -PE -CF594	BD Biosciences	562757
TER -119 -154Sm	Fluidigm	3154005C

4.13 Cytology (II)

Sorted F4/80^{Low} and F4/80^{Hi} fetal liver macrophages were spun down onto microscopic slides (Shandon cytospin III, Tecan). The cells were stained with Diff-Quick (REASTAIN) and imaged using Zeiss AxioVert 200M (Zeiss) with a Plan-Noefluar 40× /0.60 objective.

4.14 Quantitative PCR (I, II, III)

Plvap mRNA expression data from sorted CD45^{-}CD31^{+} podoplanin^{+} LECs and from CD45^{-}CD31^{+} podoplanin^{-} BECs were obtained with permission from the database of the Immunological Genome Project. (I)

Total RNA from samples was isolated using the Nucleo-Spin RNA kit (Macherey–Nagel) (II) or the RNAeasy Plus Micro kit (QIAGEN) (II, III). The RNA was reverse-transcribed to cDNA with SuperScript VILO cDNA Synthesis kit (ThermoFisher Scientific) (II) or SensiFastTM cDNA Synthesis Kit (Bioline) (III) according to the manufacturers' instructions. Quantitative PCR (qPCR) was carried out using Taqman Gene Expression Assays (ThermoFisher Scientific). The reactions were run using the 7900HT Fast Real-Time PCR System (Applied Biosystems/ ThermoFisher Scientific) (II), QuantStudio 12K Flex Real-Time PCR System (Applied Biosystems/ ThermoFisher Scientific) (II, III) or Quant Studio 3 Real-Time PCR System (Thermo Fisher Scientific) (III) at the Finnish Microarray and Sequenc-ing Centre (FMSC), Turku Centre for Biotechnology, Turku, Finland. (I) **Table 3** lists the genes that the qPCR was carried out for. Relative expression levels were calculated using Sequence Detection System (SDS) Software v2.4.1, QuantStudio 12 K Flex software, and DataAssist software (all from Applied Biosystems/ThermoFisher Scientific) (I) or Applied Biosystems® analysis modules in Thermo Fisher Cloud computing platform (ThermoFisher Scientific) (III). Results were presented as percentages of the control gene mRNA levels from the same samples.

Table 3. The genes of interest and their qPCR primers.

Gene	Primer	Pub.
Plvap	Mm00453379_m1	II
Lyve-1	Mm00475056_m1	II
Actb (control)	Mm00607939_s1	II
Cx3cr1	Mm00438354_m1	II, III
Mrc1	Mm00485148_m1	II
Adgre1/Emr1/F4/80	Mm00802529_m1	II
Itgam	Mm00434455_m1	II, III
Gata2	Mm00492301_m1	II
Flt3	Mm00439016_m1	II
Ccr2	Mm04207877_m1	II, III
Csf1r	Mm01266652_m1	III
Zbtb46	Mm00511327_m1	III
C1qa	Mm00432142_m1	III
Spi1	Mm00488142_m1	III
Itgax	Mm00498701_m1	III
Actb (control)	Mm02619580_g1	III

4.15 Electron microscopy (I, II)

4.15.1 Transmission electron microscopy (I, II)

Tissue samples were collected and fixed in 5% glutaraldehyde in 0.16 M s-collidine buffer. After 2 h post-fixation with 2% OsO4 containing 3% potassium ferrocyanide, samples were dehydrated with a series of increasing ethanol concentrations and embedded in Epoxy Embedding Medium kit (45359 Fluka). 70 nm sections were cut and stained with 1% uranyl acetate and 0.3% lead citrate. The samples were imaged with a JEOL JEM-1400 Plus transmission electron microscope.

4.15.2 Scanning electron microscopy (I)

Mice were fixed by transcardial perfusion of a solution of 2% glutaraldehyde and 4% PFA in phosphate buffer before tissue collection. The lymph nodes were washed and the capsules of the nodes were peeled off with forceps under a stereomicroscope. The samples were post-fixed for 60 min at 20 °C in 1% OsO4 in

0.1 M phosphate buffer and dehydrated through a graded ethanol series and dried with a critical point dryer (HCP-2, Hitachi). The samples were coated with 250Å gold by an ion-sputtering device (IB-3; Eiko) and examined with a scanning electron microscope (S-3000H; Hitachi) at an accelerating voltage of 25 kV.

4.15.3 Immunoelectron microscopy (I)

Mice were fixed by transcardial perfusion of cold periodate-lysine-PFA and the PLNs were collected. Samples were post-fixated with the same fixative, embedded in OCT and snap-frozen. Sections were stained by an immunogold method (15-nm gold particles) with a MECA-32 first-stage antibody. Thereafter the sections were fixed with 1% glutaraldehyde solution and 2% osmium tetroxide solution, dehydrated in graded ethanol and embedded in epoxy resin. Ultra-thin sections were stained with uranyl acetate and imaged with a JEOL JEM-1400 Plus transmission electron microscope.

4.16 Immunofluorescence staining and imaging of sections (I-III)

In general, immunofluorescence staining and imaging were performed on samples that were collected and then frozen to OCT. Sections of desired thickness were cut, acetone fixed and stained with directly conjugated antibodies and a combination of primary and secondary antibodies. Antibodies that were used are listed on **Table 4**. Stained samples were mounted in Prolong Gold with or without DAPI or the nuclei were stained with Hoechst (Thermo Fisher Scientific, Cat. 62249) and then mounted with Prolong gold without DAPI. Imaging was performed with an appropriate microscope. Images were acquired with a LSM 780 confocal microsope (Zeiss) using a Plan-Apochromat 20× /0.8 objective, a C-Apochromat 40× /1.20 W Korr M27 objective, C-Apochromat 63× /1.2 oil objective or Plan-Apochromat 100× /1.40 oil DIC objective. A spinning disk confocal microscope (Intelligent Imaging Innovations) with a Plan-Apochromat 63× /1.4, 20× /0.8, 10× /0.45 or LD c-apochromat 40× /1.1 W objective was also used. Zen 2010 software (Zeiss), SlideBook 6 software (Intelligent Imaging Innovations, Inc.) Imaris 8.0 software (Bitplane) and ImageJ software were used. Subtraction of background was used equally for all images. In certain images, brightness was changed in a linear way with ImageJ software. Brightness adjustments and thresholds were always applied equally to images that were compared. For certain representative images, maximal projection was used to generate z-stacks and noise was reduced with the mean filter in ImageJ software.

Table 4. Antibodies used in immunofluorescence microscopy

Antibody	Company	Cat#	Pub.
αSMA-Cy3	Sigma	C6198	I
αSMA-FITC	Sigma	F3777-2ML	III
B220-Pacific Blue	BD Biosciences	558108	I
CD4-A647	BD Biosciences	557681	I
CD8-A488	BD Biosciences	557668	I
CD11b-eFluor450	eBiosciences	48-0112-80	I
CD11c-A488	eBiosciences	53-0114-82	I
CD31-A488	BioLegend	102514	I, III
CD31-APC	BioLegend	102510	I, II
CD206-A488	Bio-Rad	MCA2235A488T	III
CD206-A647	BD Biosciences	565250	III
F4/80-A488	Invitrogen	MF48020	II
Meca-32-A488	BioLegend	120506	I
Meca-32-A647	Serotec	MCA2539A647	I
Siglec - F -A647	BD Biosciences	562680	III
Phalloidin-A546	Life Technologies	A22283	I
Rat IgG2a-A488	BioLegend	400525	I
AOC3	Harlan Sera-Lab	PAS-2542	I
CD31	BD Biosciences	550274	II
CD31	BD Biosciences	553370	III
CD54 (ICAM)	BD Biosciences	550287	I
CD106 (VCAM)	BD Biosciences	550547	I
CD117	BD Biosciences	553352	II
CD144 (VE-cadherin)	Pharmingen/BD Biosciences	550548	I, III
CD169	Serotec	MCA884EL	I
Caveolin	Santa-Cruz	sc-894	I, II
CCRL1	Santa Cruz	sc-46835	I
Collagen I	Chemicon	AB765P	I
ER-TR7	Abcam	ab51824	I
ER-TR7-DyLight 405	Novus Biologicals	NB100-649	I
F4/80	Bio-Rad	MCA497R	III
Lyve-1	Reliatech	102-PA50AG/103-PA50	I, II
MAdCAM-1	E.Butcher Standford		I, II
MECA-32/Plvap	Invivo	MECA32-17-8-AK 2028/01	II
MECA-32/Plvap	BD Biosciences	550563	I, II

Antibody	Company	Cat#	Pub.
Mouse anti human Plvap	Abcam	ab8086	I, II
Perlecan	Acris	DM3099P	I
Podoplanin	R&D Systems	AF3244	I
Prox-1	R&D Systems	AF2727	I
Rat IgG2a	BD Biosciences	553926	I
VEGF	Abcam	AB46154	II
anti Goat IgG-A633	Molecular Probes	A21477	I
Donkey anti Goat IgG-A633	Life Technologies	A21082	I
Donkey anti Rat IgG-A488	Life Technologies	A21208	I, II
Goat anti Mouse IgG-A488	Invitrogen	A11029	II
Goat anti Rat IgG-A546	ThermoFisher	A11081	I-III
Goat anti Rat IgG-A647	Life Technologies	A21247	I, II
Goat anti Rabbit IgG- A405	Life Technologies	A31556	I
Goat anti Rabbit IgG-A546	Invitrogen	A11035	II
Goat anti Rabbit IgG-A633	Invitrogen	A21071	I, II

4.16.1 Visualization of luminal PLVAP (I, II)

For visualization of the luminal location of PLVAP, unconjugated or A488-conjugated MECA-32 or control antibody was administered to recipient. Injections were made either s.c to hind paw (I) or directly to the umbilical and vitelline veins of the yolk sac of E12.5 embryos (II). After 5 min (I) or 1 min (II) circulation, the recipients were killed and tissues of interest were collected, processed for microscopy.

4.16.2 Whole-mount (I, II)

Afferent lymphatics were visualized by staining the mechanically separated dorsal half of a WT ear with antibodies, followed by extensive washing and imaging the next day. The whole-mount staining methodology for PLNs, YS and AGM was adapted from a published protocol (Yokomizo et al.2012). Samples were imaged and three–dimensional reconstructions were generated from z-stacks, the results were converted to QuickTime files with Imaris 8.0 software (Bitplane).

4.16.3 PLVAP expression in human samples (I, II)

PLVAP expression in human tissues was investigated in lymph nodes and liver. Peripheral lymph nodes which were macroscopically and microscopically normal, originated from surplus tissue from head and neck surgery. Human fetal liver sample from pregnancy week 18 was used for human liver analysis. Samples were sectioned and stained to determine the PLVAP expression and location using microscopy.

4.17 Histology (II)

Formalin-fixed, paraffin-embedded sections of embryo livers were cut, deparaffinized before antigen retrieval. Endogenous peroxide was quenched with 3% H_2O_2 and non-specific immunoglobulin binding was blocked with rabbit serum. Endogenous biotin and avidin were blocked using DakoCytomation Biotin blocking system (Dako, X0590). Biotin–avidin complexes with the secondary antibodies were formed with using Vectastain ABC kit (PK-6100, Vector Laboratories). Liquid DAB+ substrate Chromogen System (Dako K3468) was used to detect the peroxidase complexes. Samples were stained with haematoxylin, dehydrated and mounted before imaging.

4.18 Iron recycling (II)

Prussian blue stainin on PFA-fixed, paraffin embedded sections was used to detect the ferric iron. The sections were counter-stained with Nuclear Fast Red. The red pulp areas of the spleen containing the blue-stained $Fe3^{+}$-containing cells were analyzed using image thresholding. In livers, whole sections were analyzed. ImageJ software was used to measure the area fractions.

4.19 Mammary gland ductal branching (II, III)

The fourth mammary gland was mounted onto a glass slide and fixed in Carnoy's fixative overnight at +4 °C or for 4 h at room temperature. The sample was rehydrated and stained with carmine alum (STEMCELL technologies, cat 07070) according to the manufacturer's instructions. The samples were dehydrated, cleared in xylene for 2–3 days, and mounted with DPX Mountant (Sigma-Aldrich). Imaging was performed with Zeiss SteREO Lumar V12 stereo microscope using NeoLumar 0.8× objective (II) or Axiovert M200 microscope using a 5× /0.25 objective (III). The mammary gland ductal tree area and the number of ductal branches were tracked manually and quantified using ImageJ with 'Skeletonize2D/3D' and 'AnalyzeSkeleton' plugins.

4.20 Statistical analyses (I-III)

Sample sizes were based on pilot analyses and published literature. Because mice of distinct genotypes were used, no specific randomization methods were implemented. During the experimental procedures with embryos, investigators were blinded to the genotypes, no other blinding methods were used. Numeric data are given as mean ± SEM. Statistical analyses were performed using GraphPad Prism software v6 and SAS 9.4. Comparisons between the genotypes were done with the Mann-Whitney or Kruskal-Wallis test. P values of <0.05 were considered statistically significant.

5 Results

5.1 PLVAP in lymphatic endothelium controls the entrance of lymphocytes and antigens into the lymph nodes

5.1.1 Deletion of PLVAP leads to abnormal lymph nodes

To study the expression and role of PLVAP in lymphatics, a *Plvap*$^{-/-}$ knockout mouse model was generated. A heterozygous × heterozygous breeding and a mixed background approach were necessary to ensure that the offspring were born alive and that approximately 15% of the expected frequency of homozygous pups survived until early adulthood. Deletion of the PLVAP expression was checked with immunoblotting and by staining blood endothelium in the peripheral lymph nodes (PLN) with MECA-32 (monoclonal antibody to PLVAP). (I: Fig 1a, S1d) Comparison to sex matched WT littermates confirmed that the PLVAP protein was successfully ablated from the knockout mice.

PLN:s from the *Plvap*$^{-/-}$ mice were unmistakably different compared to the WT PLN:s. Enlarged B cell follicles were visible with the microscope and further studies with flow cytometry showed a normal total lymphocyte count, but reduced amounts of $CD4^+$ and $CD8^+$ T cells and an increase of $B220^+$ B cells. (I: Fig 1b-d) More B cells positive for the germinal center activation marker GL7 was seen in addition to a larger number of activated $CD25^+$ T cells and $CD44^{Hi}CD62L^{Low}$ T cells among $CD3^+$ cells in the *Plvap*$^{-/-}$ mice. (I: Fig 1e-g) However, even in the absence of PLVAP, the lymphatic vasculature in the PLN seemed normal. (I: Fig 2a, 2g, 2h) Also the distribution and composition of the reticular conduit network which travels along the T cell areas were normal, although the area was diminished in size, due to the enlarged B cell follicles. (I: Fig 4i, S5c-f)

5.1.2 WT subcapsular sinus LECs express PLVAP

A closer look at the subcapsular sinus of the WT PLN revealed a clear PLVAP signal in the sinus lining LECs, both on the floor and ceiling of the sinus. (I: Fig 2a, 3a) To confirm the unexpected finding of the presumably BEC-specific molecule,

several approaches were used. Detection of PLVAP in the draining lymph node SCS after s.c administration of MECA-32-A488, together with immunoelectron microscopy corroborated the finding. (I: Fig 2e, 3e-f,) A comparison of mRNA expression in the BECs and LECs from WT PLN showed obvious transcription of *Plvap* in the LECs, albeit the level being lower in the LECs. (I: Fig 2b, 2f) Finally, human PLN also showed expression of PLVAP in the subcapsular sinus. (I: Fig S3c) No PLVAP signal was found in the parenchyma of the WT PLN, excluding the blood vascular endothelial cells. Also, the afferent lymphatics were devoid of PLVAP expression. (I: Fig 2i, S3e, S3f) Much like in the BECs, PLVAP formed diaphragms in the endothelium, especially in the sinus floor that is in close contact with the underlying parenchymal conduits. (I: Fig 3b) The diaphragms were found on TECs and caveolae. Even though the *Plvap*$^{-/-}$ mice lacked the diaphragms, the endothelial structures could still be found on the LECs. (I: Fig 3c-f, S4h-i)

5.1.3 Lack of PLVAP leads to increased antigen entry and enhanced B & T cell activation in the PLN

The open-ended endothelial channels of the LEC floor in *Plvap*$^{-/-}$ mice are in close proximity of the conduit network, therefore the effect of the missing diaphragms was investigated. Fluorescently-labeled molecules (dextrans) with different molecular weights (10 kDa, 70 kDa, 500 kDa) were injected s.c into mouse foot pad and the conduit filling was analyzed. Normally, molecules of 70 kDa and larger molecular weight are excluded from entering the conduit network. In the *Plvap*$^{-/-}$ mice, the smallest molecule crowded the conduits instantly and the 70 kDa and even 500 kDa molecules entered the network. (I: Fig 4a-d) In the absence of PLVAP filters, also a large protein antigen (180 kDa enzyme AOC3) reached the parenchyma readily. There, the antigen was taken up by the parenchymal macrophages and DCs, which act as antigen presenting cells in the lymph node. (I: Fig 4c, 4f, 4h)

To determine whether the increased antigen-entry was PLVAP-dependent, we ruled out some other possibilities. Caveolae are known to facilitate trans-endothelial passage. (Schubert et al. 2001) Our studies on the *Cav*$^{-/-}$ mice revealed that the PLVAP expression in the SCS LECs was comparable to WT. This indicated that the majority of PLVAP is located in non-caveolar structures. Also the results from the dextran experiments were similar to WT. (I Fig 5a-c) Macrophages participate in antigen transfer from the sinus to the B cell follicles. (Junt et al. 2007) To test the macrophage involvement in the antigen transfer into the conduits and parenchyma, macrophages were depleted by using clodronate containing liposomes. Macrophage-ingested liposomes release the clodronate, a bisphosphonate drug, which induces apoptosis of the phagocyte. (Van Rooijen and

Sanders 1994) Depletion of PLN parenchymal CD169^{+} F4/80^{+} and sinus-lining CD169^{+} F4/80^{-} macrophages (both PLVAP negative) using clodronate-containing liposomes did not affect the PLVAP expression of the LECs, nor the result of the dextran assay, i.e. the 70 kDa and 500 kDa molecules were excluded from the parenchyma. (I: Fig 5f, S6i) Thus, PLVAP in the LECs regulates the entry of lymph borne molecules to the PLN parenchyma, in a size-dependent manner.

5.1.4 Lymphocytes use PLVAP to transmigrate through the lymphatic endothelium

To study whether the PLVAP diaphragms of the SCS floor affected the migration of lymph borne lymphocytes to the parenchyma, lymphocytes from WT donors were labeled with a green cytosolic dye CFSE, injected s.c into *Plvap*$^{-/-}$ and WT mice and left to migrate to the draining lymph node. In the absence of PLVAP, the transferred lymphocytes entered the lymph node in greater numbers in comparison to WT. Blocking the PLVAP filter with a conjugated or unconjugated MECA-32 antibody led to reduced entry of lymphocytes into the lymph node. (I: Fig 6a-c) Electron microscopy analyses showed that the interaction between lymphocytes and LECs typically took place where cell surface structures such as caveola and TECs located. *In vitro* -data revealed that 32,3 % ± 5,3% (mean ± sem) of the lymphocytes traversed the monolayer of LECs via the transcellular route. Immunofluorescence stainings showed the lymphocytes extending F-actin^{+} podosomes to probe the LEC membrane and pushing aside the PLVAP and F-actin. PLVAP^{+} areas were sporadically situated on the peripheral cytoplasm and seemed to coincide with F-actin^{Low} expression, but were almost absent in the cell-cell junction areas. (I: Fig 7a-f, S7e-g) These data suggest that PLVAP on the endothelium facilitates transcellular migration.

5.2 PLVAP regulates the establishment of tissue-resident macrophage populations during development

Studying the immune system and immune cells of the adult *Plvap*$^{-/-}$ mice revealed that the absence of PLVAP led to unexpected perturbations in the macrophage system. Several tissues lacked macrophages to some extent or almost totally. F4/80 is often used to differentiate the fetal-derived tissue-resident macrophages (F4/80^{Hi}) from monocyte-derived macrophages (F4/80^{Low}). (Schulz et al. 2012, Hashimoto et al. 2013) Therefore we used F4/80 (among other markers) to study the macrophage biology of the *Plvap*$^{-/-}$ mouse.

5.2.1 Deletion of PLVAP leads to decrease in F4/80^{Hi} tissue-resident macrophage populations in the adult mice

Flow cytometry analyses showed that several tissues had significant reductions (75%-95%) in embryonic-derived tissue-resident macrophage populations, but normal or slightly increased amounts of bone marrow –derived tissue macrophages. (II: Fig1a) In the adult mice, the production of monocytes and macrophages seemed normal, determined by the fact that frequencies of hematopoietic stem cells (HSC), common myeloid and common lymphoid progenitor cells were comparable to WT mice. In addition, the frequencies of bone marrow and blood monocytes were normal, as were the amount of recently entered tissue monocytes in the spleen and liver. (II: Fig 1a, S2b-f) Frequencies of B cells and T cells were not affected by the PLVAP deletion, except in the PLNs. (I: Fig 1d, II: Fig S2g, S3a-b)

5.2.2 Decreased fetal liver -derived, but not YS-derived macrophages in tissues of *Plvap*$^{-/-}$ mice

Tissue-resident macrophages originate from the fetal era, so the *Plvap*$^{-/-}$ mice were studied at an embryonic time point. Flow cytometric analyses showed that at E16.5 the YS-derived macrophage counts in the spleen and lung were not influenced by the lack of PLVAP. Also the number erythro-myeloid progenitors (EMP) and the colony forming ability of E10.5 YS cells remained unaffected, as did the microglia of both embryonic and adult mice. (II: Fig 2a, S4b-e) However, the frequency of liver-derived macrophages in the embryonic spleen and lung were significantly reduced. The blood also had significantly fewer liver-derived monocytes. (II: Fig 1b)

5.2.3 Macrophage precursors accumulate in fetal liver

Since the production of fetal macrophages shifts to the liver at E12.5, a closer look at the *Plvap*$^{-/-}$ liver was taken. The lack of PLVAP (which is synthesized in WT liver endothelium from E11.5 onwards) did not disturb the overall morphology and vasculature of the embryonic liver. (II Fig: 3a-b S7a-c) Flow cytometric analysis revealed that the amount of YS-derived macrophages in the liver remained unaffected at E12.5-E16.5, but at the same time a clear accumulation of liver-derived macrophages and especially liver monocytes was seen. (II: Fig 2b-d, S5c) The frequencies of monocyte progenitor cell types were comparable between the *Plvap*$^{-/-}$ and WT livers, as were the frequencies of EMPs and HSCs (and their colony forming abilities at E12.5). (II: Fig S5c-f) Tamoxifen inducible *Plvap*$^{F/F}$*; CAGCre-ER*^{TM} and conditional *Plvap*$^{F/F}$*; Lyve-1Cre-* mouse indicated that PLVAP

expression is needed at E13.5-E14.5 for the normal egress of fetal liver monocytes. (II: Fig S8a-f)

5.2.4 PLVAP interacts with heparin and chemotactic molecules

In embryonic WT liver, PLVAP can be found on top of fenestrae, whereas in *Plvap*$^{-/-}$ liver the endothelial structures are formed normally, but remain uncovered. (II Fig 3d) We reasoned that if the open-ended fenestrae leave cells trapped in the liver, the PLVAP diaphragms might be needed to interact with the cells, essentially guiding them out. A series of interaction studies with molecules of interest, based on the literature, were conducted. PLVAP (in certain experiments endogenous PLVAP from E14.5 liver and in others a PLVAP-IgG Fc fusion protein) bound to heparin. PLVAP also bound to the chemotactic molecules VEGF-A and neuropilin-1, but only in the presence of heparin. (II Fig 3e-h, S9a-e) We also discovered that in the liver, common monocyte progenitors and Ly6C^{+} and Ly6C^{-} monocytes express receptors for VEGF-A and neuropilin-1. (II: Fig S9f-i) This supported our theory that the PLVAP may immobilize chemotactic molecules which are recognized by fetal monocytes.

5.2.5 Deficiency of fetal-derived macrophages leads to Fe^{3+} accumulation and altered mammary branching in adult *Plvap*$^{-/-}$ mice

The deficiency of F4/80^{Hi} macrophages in the *Plvap*$^{-/-}$ mice was evident in the spleen and liver of adult mice. (II: Fig 1a, 2b) To investigate if bone marrow -derived macrophages can take over the tissue-resident macrophage functions, the recycling of iron was studied. Prussian blue staining showed that in the *Plvap*$^{-/-}$ mice Fe^{3+} accumulated in the red pulp of the spleen and in the liver. (II: Fig 4a-b, S10c) Mammary gland branching is partly macrophage dependent and was studied in the prepubertal 4,5wk old *Plvap*$^{-/-}$ female mice. F4/80^{+} macrophage counts within the 4th inguinal mammary glands were reduced, while the other lymphocyte counts remained unaffected. The ductal branching was significantly stunted in the otherwise proportionally sized mammary gland. (II: Fig 4c-f, S10d-e, III: Fig 5a) These results showed that the adult monocyte-derived macrophages cannot fully compensate the tissue-resident macrophage functions in the *Plvap*$^{-/-}$ mice.

5.3 Fetal-derived macrophages occupy mammary gland throughout adulthood

The striking under-development of the mammary gland ductal network in the *Plvap*$^{-/-}$ mice prompted us to perform a more detailed investigation of the mammary gland macrophage biology. Steady state mammary tissue macrophage status has not been thoroughly investigated, as much of the research focuses in pathological tumor associated macrophages.

5.3.1 Macrophages reside in the mammary gland already before birth

Flow cytometric analyses revealed that F4/80^{+} cells of the 5wk WT mammary gland can in fact be divided to F4/80^{Hi} and F4/80^{Int} populations. (II: Fig 4c, III: Fig 1a) Gene profiling and surface marker analysis of the two mammary gland populations supported the different phenotypes of the macrophages. (III: Fig S2c-d) Two robust populations of macrophages were found in the mammary gland at all studied postnatal time points (1wk, 2wk, 5wk and 3 months of ages), with the F4/80^{Hi} macrophages composing approx. 50% of total mammary gland macrophages. Interestingly, two macrophage populations were present in the mammary gland already before and right after birth. This, in addition to reduced macrophage counts in the *Plvap*$^{-/-}$ mammary gland (see 5.2.5) suggested YS-derived and fetal liver -derived origins to the mammary gland tissue-resident macrophages in adulthood. (III: Fig 1a, S2a-b, h)

5.3.2 Fetal liver -derived macrophages seem to constitute the majority of tissue-resident macrophage pool in mammary gland

To further dissect the origin of the embryonic macrophages in the adult mammary gland, several mouse models and experimental settings were utilized. Experiments with anti CD115 antibody (which blocks the production of YS-derived macrophages) showed that the YS-derived macrophages fall into the F4/80^{Hi} population in E17.5 WT mice, but the depletion did not affect the mammary gland macrophage populations significantly in later time points. Analysis of the mammary glands of CX3CR1-YFP and CSF1R-YFP -reporter mice (which label the YS-derived macrophages/progenitors and their progeny) showed that the YFP^{+} cells were also F4/80^{Hi} in all time points and that YFP^{+} F4/80^{Hi} fetal-derived macrophages persisted in the adult mammary gland. *Plvap*$^{-/-}$ mice, which have a deficiency of fetal liver -derived macrophages, but normal YS-derived macrophages, have a small proportion (12% vs 39% in the WT) of F4/80^{Hi}

macrophages in the mammary gland. This small population is most probably from YS origin. (III: Fig 1b-d, S2g) These results suggest that the majority of F4/80^{Hi} macrophages in the adult mammary gland are originating from the fetal liver, rather than the YS.

5.3.3 CCR2-independent migration of macrophages to mammary gland

To investigate the contribution of adult monocytes to the F4/80^{Int} macrophage population in the mammary gland, we studied two commonly used knock out mouse models with reductions in mature Ly6C+ and circulating Ly6C- monocytes. In breast cancer, monocytes are recruited to the tumor in CCR2-dependent manner. (Bonapace et al. 2014) Flow cytometry analysis of Nur77$^{-/-}$ mice mammary glands showed no reduction of mammary gland monocytes and Ccr2$^{-/-}$ mice showed only a modest reduction in the F4/80^{Int} population of macrophages. (III: Fig 2a, S3d) These results suggest that in a healthy mouse the majority of adult monocyte -derived macrophages migrate to the mammary in a CCR2-independent manner.

5.3.4 CD206 as a putative marker to define fetal-derived macrophages in mammary gland

Distinguishing the tissue-resident macrophages often relies on the common macrophage surface marker F4/80. However, after analyzing the mammary gland macrophages with mass cytometry, we observed that CD206 is actually more powerful at phenotypically differentiating the fetal-derived and adult monocyte-derived macrophages in the mammary gland. F4/80^{Hi} macrophages were CD206^{Hi} and the deficiency of CD206^{Hi} macrophages in *Plvap*$^{-/-}$ mice and in clodronate treated mice after recovery imply that CD206 does identify fetal macrophages. Also, CD206^{Hi} macrophages expressed high levels of typical macrophage markers (CD64, F4/80, Siglec-1) in flow cytometric and qPCR analyses. (III: Fig 3d, 4b)

5.3.5 CD206^{+} macrophages are associated with ductal branching and scavenging in mammary gland

Macrophages are associated with ductal branching morphogenesis of the mammary gland. Mammary gland whole mount stainings showed that the ductal branching was comparable to WT mice in the knockout models with defective bone marrow monocyte migration (*Ccr2*$^{-/-}$ and *Nur77*$^{-/-}$). However, the ductal morphogenesis failed almost completely in the embryonic macrophage -deficient mice (*Plvap*$^{-/-}$ mice, which have normal YS-derived macrophages, see 5.2.2). (III: Fig 5a)

In addition to assisting in ductal branching, macrophages are known scavengers in many tissues. We studied the scavenging abilities of the mammary macrophages and the $CD206^{Hi}$ macrophages showed superior scavenging properties in assays where dextran, acetylated LDL or immunocomplexes were injected intravenously (i.v) into WT mice. The injected particles were taken up by the $CD206^{Hi}$ macrophages much more readily in comparison to the monocyte-derived $CD206^{Neg/low}$ macrophages. (III: Fig 5b) Immunofluorescence stainings showed that the $CD206^{+}$ macrophages resided next to the ducts and small vessels of the mammary gland, even reaching to the lumen. These locational data support their scavenging function. (III: Fig 5d-f, S7b-d) These data show that the $CD206^{+}$ macrophages possibly perform specialized functions in the mammary gland.

6 Discussion

The basis of this thesis was laid by the unexpected discovery of PLVAP in the PLN subcapsular sinus. Until then, PLVAP had been studied only in the blood vascular endothelium, where it affected endothelial permeability. We reasoned that PLVAP in the PLN might serve the same purpose and studied if the absence of PLVAP had an effect on the immunological functions of the PLN. While investigating the leukocyte populations we noticed that the *Plvap*$^{-/-}$ mice experienced reduced frequencies of macrophages in several tissues. Since the production of macrophage precursors in the adult mice seemed unaffected, we reasoned that there must be another reason for the diminished macrophage counts. Embryonic macrophages had been reported to colonize tissues and remain in them during adulthood. PLVAP-deficient mice seemed to have diminished frequencies of tissue-resident macrophages in many tissues, especially in the mammary gland. Thus the final step was to elaborate the mammary gland macrophage kinetics and inspect the origins and functions of the tissue-resident macrophages in the mammary gland. The results from this thesis underline the importance of endothelial regulation in the developing embryo and in the adult individual. This chapter discusses some of the key results and the questions they raise. At the end of this chapter I will discuss some potential approaches to utilize the discoveries in this thesis.

6.1 PLVAP expression in the lymphatics (I)

The endothelial protein PLVAP has been previously studied in the blood vasculature and has been established as a blood endothelium specific marker. (Baluk, McDonald 2008) PLVAP is the only known component of the diaphragms that overlay caveolae, TECs and fenestrae and no other localization has been found for PLVAP. (Tkachenko et al. 2012) It was surprising to detect PLVAP expression within the lymph node LECs. The immunofluorescence staining of mouse and human peripheral lymph node clearly showed that the lymphatic endothelial cells of the subcapsular and medullary sinus expressed PLVAP. Bright staining by the MECA-32 antibody in the blood vessels of the PLN combined with the blank staining in the *Plvap*$^{-/-}$ mice corroborated the reliability of the antibody used to detect PLVAP. Comparing *Plvap* mRNA expression in the LECs and BECs

showed that the LECs clearly express the mRNA, although in a lower level compared to BECs. Co-staining with lymphatic endothelial markers confirmed the PLVAP expression within the SCS. Finally, electron microscopy studies suggested the existence of the classical cartwheel-like structure that PLVAP forms in other tissues, and showed the binding of gold conjugated PLVAP antibody to the caveolae, transendothelial channel and fenestrae structures. These results prove that the PLVAP, formerly thought to be BEC specific is also expressed by the adult mouse and human PLN. The unique expression of PLVAP in the SCS LECs and the absence of PLVAP in the afferent lymphatics may point to a specific filtering function of the SCS LECs. It is likely that the expression has gone unnoticed by researchers because of the lower expression and specific location in the SCS, relatively far from the BEC vessels which might be stained with MECA-32 for their blood vessel identity.

6.2 PLVAP mediated regulation of antigen and leukocyte migration (I, II)

6.2.1 Antigen migration (I)

The lymphatic expression of PLVAP was focused on the SCS floor and the absence of expression in the afferent lymphatics led us to hypothesize that the diaphragm could serve the same function in the LECs as it does in the blood endothelium. Indeed, in the absence of PLVAP, the entry of small and large antigens was increased. Several approaches were used to prove that the antigen entry is regulated by PLVAP in the LECs. We showed that once the larger (>70 kDa) molecules gain access through the SCS, like in the case of the *Plvap*$^{-/-}$ mice, they are non-discriminatively transported onwards in the conduit system. Our data are supported by the results from Thierry et al. who showed that the conduit system is able to transport locally produced large IgM molecules, but they cannot enter the LN parenchyma from the blood or lymph, i.e. cross the endothelium layers (Thierry et al. 2018). We used Cav$^{-/-}$ mice to show that the endothelial caveolae, which are known to facilitate molecule transport, had no effect on the antigen entry to the PLN parenchyma. Similarly we showed that the sinus lining macrophages, which are known to transport larger antigens trough the SCS (Junt et al. 2007), did not affect the conduit filling. The endothelial cells are known to endocytose particles and the amount of endothelium-facilitated transport was not quantified. However, the efficacy of endothelial transport is small compared to macrophages (Anselmo and Mitragotri 2017) and since the macrophages had no significant effect on dextran transport, it is unlikely that the LEC transcytosis would account for the robust dextran migration to the conduits. These data support our

interpretation that the PLVAP diaphragms on LECs have a decisive role in regulating the entry of the molecules to the parenchyma.

6.2.2 Leukocyte migration (I, II)

Leukocytes can traverse endothelial layers via the transcellular or paracellular route. (Carman et al. 2007) The results on CFSE –labeled leukocyte migration showed that the absence of PLVAP diaphragms on the SCS led to increased $CD4^+$ T lymphocyte (and $B220^+$ B cells, but less so compared to the T cells) entry into the parenchyma of the PLN, similarly to the increased antigen entry. Blocking of the diaphragm in WT mice using a MECA-32-antibody in turn decreased the lymphocyte entry. These results indicate that in steady state, the migrating lymphocytes interact with the PLVAP filter and perhaps use it as a sign of a route with less resistance.

Labeling of the lymphocytes with CFSE even at recommended concentrations has been shown to negatively affect the migration of B cells to the PLN *in vivo*, but not to affect their ability to respond to chemotactic signals or bind to HEVs *in vitro*. (Nolte et al. 2004) We showed an increase of CFSE-labeled lymphocytes in *Plvap*$^{-/-}$ PLN in comparison to WT, and the blocking of PLVAP with an antibody reduced the influx of both T and B cells. PLVAP interactions with chemotactic molecules are possibly needed for the monocyte egress in the fetal liver, but we did not check the presence of chemokines in the PLN. Thus, blocking of the PLVAP diaphragm with an antibody might have hampered the transmigration by preventing the migrating lymphocytes from reaching the diaphragm-bound chemokines. CFSE-labeling of the cells, rather than differing migration mechanisms between the T and B cells, might explain the underrepresentation of $B220^+$ cells in the migrated lymphocytes.

Our results support the *in vitro* data from Keuschnigg et al. which shows that PLVAP is important for the transmigration, more so than for the paracellular migration of lymphocytes. (Keuschnigg et al. 2009) Migrating cells made contact with $PLVAP^+$ areas which were also F-$actin^{Low}$. The PLVAP covered TECs and fenestra are structures that in essence create a tunnel through the endothelial cells and often localize to the thinnest, peripheral part of the cytoplasm. This might provide the lymphocyte a sign of easy entry into the parenchyma via the transcellular route. Interestingly, the lymphocytes are too big to fit through the TECs and fenestra, so it is likely that some remodeling of the endothelial architecture takes place. Leukocytes have been shown to extend podosomes to palpate endothelial cell surface and induce and expand pores to facilitate migration across the endothelium. (Carman et al. 2007) The same techniques could be used to push aside or degrade the PLVAP diaphragm and open the pre-existing pore to fit

through. **Figure 5.** summarizes the role of PLVAP in antigen and lymphocyte migration from the SCS to the parenchyma of the PLN.

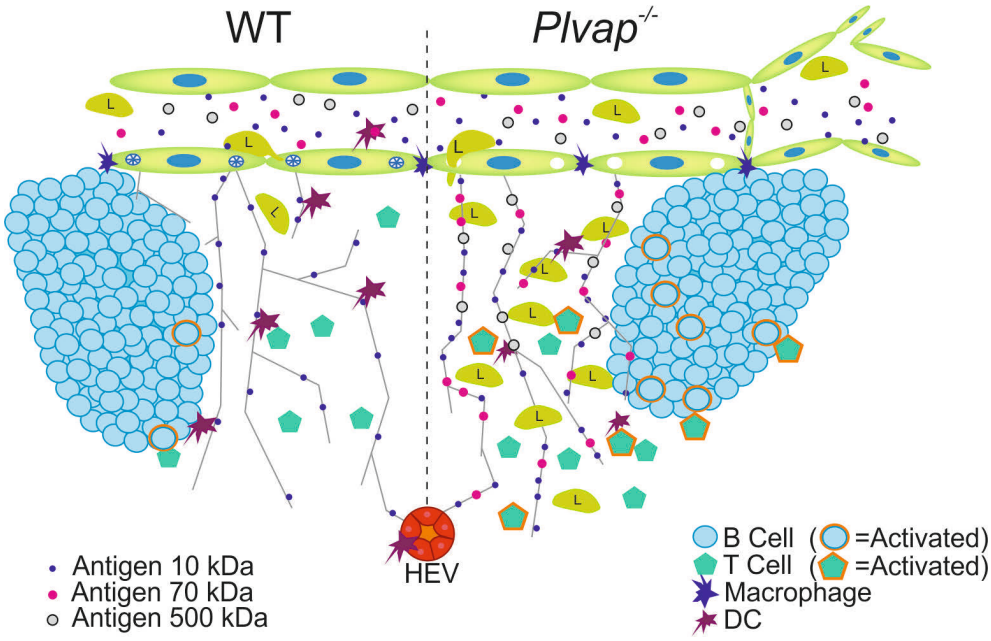

Figure 5. Summary of the effects of PLVAP in migration of lymphocytes and various sized antigens from the SCS to PLN parenchyma. L= Lymphocyte

However, contrary to the increased lymphocyte migration into the PLN (Publication I), the absence of PLVAP filter in the fetal liver led to decreased migration of macrophage precursors to the blood stream (Publication II). We and others have shown that PLVAP interacts with molecules such as VEGF-A and neuropilin-1. (Keuschnigg et al. 2012) Our results showed for the first time the interaction between heparin, neuropilin-1, VEGF and PLVAP and that they potentially interact with the neuropilin-1 and VEGFR1 in the fetal monocytes, guiding the monocytes into the blood stream. The molecular interactions offer a possible explanation for the monocyte accumulation within the fetal liver in the absence of PLVAP diaphragm (and the subsequent presence of open fenestrae).

Nevertheless, it is unexpected that the absence of PLVAP filter in the SCS and fetal liver affects the migration cascade in opposite ways, as the absence of PLVAP has so far only been linked to increased leakiness in all tissues. No other structure or function has been reported for PLVAP besides the cart wheel -like diaphragm and the gene is well conserved in mouse, rat and human tissues. (Stan et al. 2001) Therefore it is unlikely that the PLVAP-diaphragm itself would function differently

in PLN and fetal liver. Possible explanations for the opposite effects of absent PLVAP on cell migration in the PLN and fetal liver might be:

Perturbed endothelial cell barrier: The SCS LEC floor could be leaky due to the lack of PLVAP or experimental procedures during the cell migration assays, allowing the labeled lymphocytes to leak through the SCS. The injection of CFSE-labeled lymphocytes is not strictly physiological, but others have shown that the injection of cells in the periphery (although intralymphatic) does not alter the lymph node structure. (Brown et al. 2018) The immunofluorescent stainings also showed that the lack of PLVAP did not disturb the overall morphology of either the PLN or the fetal liver suggesting it is unlikely that the increased cell migration would be the result of perturbed morphology in the PLN.

Differences in migration routes: The migrating lymphocytes and fetal monocytes utilized different routes to migrate to their target tissues (lymphatics in Publication I and blood vessels in Publication II). PLVAP is widely expressed in the blood vasculature (Stan et al. 2001) and the systemic absence of PLVAP could result in leaky vessels outside the liver which would not be able to facilitate fetal monocyte migration from the liver to the target tissues. In the lymphatics, PLVAP expression has not been reported in the periphery.

Differences in migrating cells: The migrating cells in Publication I were CFSE-labeled adult lymphocytes as opposed to the endogenous, non-manipulated fetal monocytes in their native environment in Publication II. The two cell types might use differing migration strategies. It is possible that the dependency of a certain migration molecule changes between fetal and adult cells. For example, adult monocytes typically migrate in a CCR2-dependent manner, whereas fetal monocytes have a more progenitor-like phenotype and migrate in a CCR2-independent manner. (Serbina, Pamer 2006) Similarly, adult lymphocytes could be less dependent on PLVAP or PLVAP-associated migration molecules.

Elucidating the reason for the opposite outcomes for cell migration in the adult lymph node and fetal liver requires more investigation of the differences in the endothelial cells. However, investigation of cell migration should preferably be conducted in a three-dimensional model. It is challenging to draw conclusions of cell location in a tissue, based on two-dimensional sections. Nonetheless it seems that PLVAP, which so far has mainly been reported to act as a filter, has the ability to participate in molecular interactions with chemokines and migrating cells. The blocking antibody experiments in PLN suggest that PLVAP is needed for the normal migration of cells in the PLN as well as the fetal liver.

6.3 PLVAP expression in humans (I, II)

Others and we have showed that PLVAP is expressed in the human PLN, in addition to several other healthy and diseased tissues. (Stan et al. 2001) The reports on PLVAP expression in human liver have been inconclusive. Strickland et al. reported that they could not detect expression of PLVAP in human fetal liver. (Strickland et al. 2005) We however, did see a clear PLVAP expression in the human fetal liver at pregnancy week 18. Our report supports the report by Stan et al. which shows that *Plvap* mRNA is expressed in the liver (and many other tissues, most abundantly in the kidney and spleen and none in the brain). (Stan et al. 2001) Strickland et al. did not specify the age of the fetus or the method of detection, so the discrepancy might be due to the use of a fetal liver from a different stage of development, analysis of a different localization, differences in antibodies or the method of detection.

6.4 Tissue-resident macrophages (II, III)

So far, reports on adult tissue-resident macrophages have mainly focused on the differences of monocyte-derived macrophages and embryonic macrophages, grouping together the YS and fetal liver -derived macrophages. One reason for this is likely the scarcity of tools to differentiate the two fetal macrophages from each other in the adult tissues. Today, reporter mice are widely used. Others and we have used an inducible YFP-reporter mouse under a CSF1R-promoter to track YS-derived cells and under a CX3CR1- promoter to follow both the YS and fetal liver -derived cells. The drawback of the tamoxifen inducible models is that the amount of labeled cells is small, because the labeling efficiency with tamoxifen is often relatively low. (Yona et al. 2013) Further studies are needed to ascertain that the macrophage effect is not related to other perturbations in the *Plvap*$^{-/-}$ mice. However, it is possible that the *Plvap*$^{-/-}$ mice could provide a useful tool to study the functional effects of tissue-resident F4/80^{Hi} macrophages.

The complete lack of PLVAP leads to a severe phenotype and the subsequent low birth rate and short life span of the pups. To counteract the adverse effects of systemic PLVAP deletion, we developed a conditional *Plvap*$^{F/F}$*; Lyve-1-Cre* knockout. Lyve-1 is expressed in the yolk sac blood vessels at E9.5-E18.5 and in the fetal liver sinusoidal endothelium from E9.5 forward (Nonaka et al. 2007, Gordon et al. 2008), so we achieved a selective deletion of *Plvap* only in Lyve-1^{+} cells but not the majority of other vessels in the liver. Also, a tamoxifen inducible model *Plvap*$^{F/F}$*; CAGCre-ER*^{TM} was generated to delete *Plvap* and both were useful in determining the crucial time window for the PLVAP expression during development for Publication II. However, Lyve-1 is expressed in many blood vessels of the embryo and not exclusively in the liver so another promoter would

be more suitable for studying the effects of sinusoidal PLVAP expression. So far, there are no reports of an exclusively liver endothelium specific promoter, so developing a liver specific PLVAP knock out mouse is not plausible. Also, the timed deletion of PLVAP in the *Plvap*$^{F/F}$*; CAGCre-ER*^{TM} only inhibits the formation of new PLVAP for a short period but does not remove the existing protein, so the effects remain modest. In the future, a mouse model which lacks PLVAP exclusively and completely in the liver endothelium could be useful for studying the functional consequences of tissue macrophage reduction.

6.4.1 Macrophages are found in the prenatal mammary gland (III)

The significant deficiency of macrophages in the *Plvap*$^{-/-}$ mammary gland prompted us to further study the mammary gland. Most mammary gland studies are performed on the 4th inguinal mammary glands. Due to ease of extraction and comparativeness with results from others, only the 4th inguinal mammary glands were used in the experiments for this thesis. Nevertheless the five pairs of mouse mammary glands are known to develop in succession and not simultaneously and to be differently differentiated. (Bolander 1990) To ensure that the macrophage phenotype that we reported was not unique to only the 4th mammary gland, we also analyzed the macrophage statuses of the 2nd, 3rd and 5th mammary glands. The different mammary gland pairs closely resemble each other in macrophage composition, suggesting a conserved role for macrophages in the mammary glands and corroborating the use of the 4th mammary gland for the mammary macrophage studies.

Macrophage presence in the WT mammary gland has not been fully elucidated during the different stages of development. In steady state macrophages have been reported in the mammary gland beginning from 2wk of age. (Gouon-Evans et al. 2000) We used the sensitive FACS method to show that two populations of macrophages can be found in the mammary gland fat pad precursor tissue already before birth at E16.5. Macrophages are often found in the niches of developing organs, as has been demonstrated in the PLN development. The inguinal lymph node develops in the area where the 4th inguinal mammary gland also forms during pregnancy. At E15.5 the inguinal lymph node starts to form and at E18.5 macrophages are found adjacent to the PLN bud. (Bovay et al. 2018) We did not remove the PLN bud for the E16.5 analyses as the PLN was too small. Theoretically, at E16.5 the macrophages in our mammary gland analyses could originate from the PLN development even though the macrophage numbers are considerable. However, the PLN was removed from the newborn mammary gland (and from all time points after that) and still the macrophage status of the breast

tissue closely resembled the E16.5 status. Our results provide information about the early development of the mammary gland cell composition and show that the mammary gland harbors sizeable macrophage populations already before birth. Our FACS analyses and immunohistochemical stainings compliment and further elaborate the data from adult mice reported by Gouon-Evans et al. and Chua et al. (Gouon-Evans et al. 2000, Chua et al. 2010) We provide an up-to-date picture of the mammary gland macrophage landscape with quantitative and kinetic data about the macrophage status within the whole mammary gland.

6.4.2 Fetal macrophages persist in the adult mammary gland (II, III)

After establishing that the prenatal mammary gland is inhabited by macrophages and the reduction in *Plvap*$^{-/-}$ mice macrophage population in the mammary gland, we wanted to investigate if the prenatal macrophages persisted in the adult mammary gland and what was their contribution to the whole mammary gland tissue macrophage population. *Plvap*$^{-/-}$ mice showed deficiencies in F4/80^{Hi} tissue-resident macrophages in many tissues, including the mammary gland. We saw two distinct macrophage populations at all stages of the development in the virgin WT mice. Chua et al. have reported F4/80^{Low} and F4/80^{Hi} macrophage populations in the 9-10 wk old mice, and our data from the 3 month old mice support their results. Chua et al. did not study the mammary gland at other ages or the developmental origin of the two populations. (Chua et al. 2010)

So far it has been thought that the adult mammary gland macrophages are derived from HSC-precursors in the bone marrow. (Varol et al. 2015) The assumption is supported by the report form Gouon-Evans et al. who showed that the total body irradiation of mice at 19 days of age reduced the macrophage counts around the TEB and led to perturbed mammary gland duct formation. Transplantation of bone marrow cell suspension rescued the blood lymphocyte counts and the ductal phenotype (Gouon-Evans et al. 2000). The experimental setting which has driven the mammary gland macrophage dogma is problematic and the results should be cautiously interpreted to steady state. Total body irradiation causes inflammation and tissue damage. (Lambert, Moores 1986) Hashimoto et al. concluded that lethal irradiation, followed by a bone marrow transplantation results in tissue macrophages being repopulated by monocytes, but the same does not happen if macrophages are depleted using other methods which cause no damage to the tissue environment. (Hashimoto et al. 2013) Lavin et al. showed that after irradiation, the monocyte-derived cells, which have gained entry to a tissue, acquire 50-90% of the genetic imprint of the original tissue-resident macrophages. (Lavin et al. 2014) These results question the monocyte-dependent

replenishment in steady state, since the irradiation might harm the tissue environment pat the point that is physiological. Indeed, we saw no replacement of the F4/80^{Hi} macrophages after a clodronate and anti-CSF1 antibody treatment, which is not likely to damage the tissue environment. We did see an ablation of all tissue macrophages and the repopulation of bone marrow -derived macrophages, but not the tissue-resident macrophages. This would suggest that the method of clearing the tissue affects the capability of monocytes to repopulate the tissue and corroborates our results that in steady state the mammary gland hosts a robust adult monocyte-independent macrophage population which appears to originate from the fetal liver.

Mass cytometry was used to study the macrophage populations of the mammary gland, as we wondered whether a marker could be found to better differentiate the fetal and adult macrophage populations, in addition to F4/80. CD206 was found to be useful in separating the two populations. Even though the CD206$^{Neg/Low}$ and CD206^{Hi} populations remained relatively close to each other in flow cytometric analyses, using CD206 has the advantage that it is not expressed by the F4/80^{+} eosinophils, which are found in the mammary gland. (Gouon-Evans et al. 2000) Using any markers to stain and detect fetal macrophages has a level of uncertainty, as it is possible that the adult monocyte derived macrophages could be able to upregulate the same molecules. This methodological problem could be resolved with lineage tracking experiments using a powerful and selective reporter line under a suitable promoter.

6.4.3 Monocyte-derived macrophage contribution to the mammary gland macrophage pool: CCR2 involvement in the F4/80^{Low} population (III)

We showed that a proportion of the mammary gland macrophages were CD11b^{+}F4/80^{Low}, which indicated that they were derived from adult monocytes. We used animals with perturbed monocyte migration to further investigate the origin of that population. Monocytes require CCR2 to exit from the bone marrow and CCR2-dependent monocyte influx to the breast is increased in breast cancer. (Bonapace et al. 2014) We however showed that in steady state, adult *Ccr2*$^{-/-}$ mice have only a modest reduction of monocyte-derived macrophages in the mammary gland, but normal or increased tissue-resident macrophages compared to WT. Newborn *Ccr2*$^{-/-}$ mice have normal mammary macrophage populations which aligns with the results from Hoeffel et al. who show that contrary to the adult monocyte migration, fetal monocytopoiesis is not CCR2-dependent. (Hoeffel et al. 2015) This suggests that the establishment of the F4/80^{Low} macrophages is CCR2-independent and that by adulthood only a fraction of the F4/80^{Low} macrophages are

replaced by CCR2^{+} bone marrow monocytes. The rest of the F4/80^{Low} macrophages are replenished by a mechanism that is yet to be determined.

Recently, Chakarov et al. reported two monocyte-derived interstitial macrophage populations which reside in contact with nerve fibers and blood vessels. They showed that the populations originate from Ly6C^{Hi} monocytes and are identifiable by Lyve^{Hi}MHCII^{Lo} and LYVE^{Low}MHCII^{Hi} signatures and evaluated that the two are present in several tissues. (Chakarov et al. 2019) We used mass cytometry to recognize two CD45^{+}CD11b^{+}Ly6C^{Hi}CX3CR1^{+}F4/80^{+} populations which could be divided by their MHCII expression to MHCII^{Low} (monocyte population 1, MO1) and MHCII^{Hi} (monocyte population 2, MO2), possibly reflecting the two populations that Chakarov et al. reported. They report that unlike MHCII^{Low}, only MHCII^{Hi} positive cells appeared to rise from the blood monocytes and expressed *Ccr2*, which could support our data that a small portion of mammary macrophages are CCR2-dependent. Interestingly, our studies pointed towards the monocyte populations being a continuum of monocytes maturing to tissue macrophages, but Chakarov et al. reported that the interstitial macrophage populations are separate lineages which arise from tissue-recruited monocytes. We had not included Lyve-1 in our marker pattern in mass cytometry or determined the location of the MO1 or MO2 population in immunohistochemical stainings, so we cannot confirm that they are interstitial macrophages but it is possible and would require more research.

Taken together, our data suggest that in the steady state virgin mouse the major mammary macrophage population is fetal-derived and that the bone marrow -derived monocytes contribute to the mammary macrophage pool in a CCR2-independent manner. The kinetics of mammary macrophage pool in relation to the origins of the population is depicted in **Figure 6.**

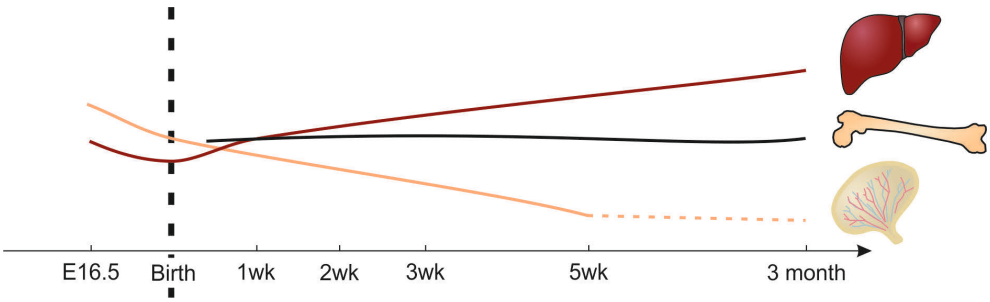

Figure 6. A relative representation of the suggested macrophage origin in the virgin mouse mammary gland at different time points. Red line = fetal liver -derived macrophages, black line = adult bone marrow -derived macrophages, orange line = yolk sac -derived macrophages

6.4.4 Hormonal effects on mammary macrophages (III)

Chua et al. reported that the macrophage abundancy in the mammary gland of 9-10wk mice changes during the estrus cycle. (Chua et al. 2010) We did not determine the estrus cycle in the adult mice, but all mice in all age points had robust macrophage counts in each experiment. The estrus cycle-associated fluctuation of the macrophage counts might explain the slightly larger variation of total cell amounts in the 3 month old mammary gland. However the frequencies of fetal and monocyte-derived macrophages stay similar in the virgin mice in every age point from prenatal to adult mice. Also, our results from male mammary tissue show comparable macrophage frequencies and phenotypes to females. These data may suggest that the macrophage composition in the mammary gland is not hormonally regulated.

6.4.5 Macrophage functions in the mammary gland (III)

Milk and milk proteins are produced by the alveoli in the mammary gland and defects in the ductal formation often lead to perturbed milk production and failed lactation. (Pollard Henninghauser 1994) Milk contains a substantial amount of macrophages, which are distinctly different from blood monocytes, highly phagocytic and linked to transmission of HIV from mother to child. (Ichikawa et al. 2003) Studying the effect of the reduced $F4/80^{Hi}CD206^{Hi}$ macrophages on milk composition would be interesting. The embryonic macrophages seem to reside in close contact with the ducts and are clearly phagocytic, so in that sense they resemble the macrophages depicted by Pollard et al. and it would be tempting to investigate whether the milk macrophages actually are embryonically-derived. Unfortunately the *Plvap*$^{-/-}$ females do not survive long enough to produce and nurse offspring, so their milk composition cannot be studied.

Interestingly, the adult male mouse mammary gland fat pads, which neither form ducts or alveoli nor produce milk, have comparable macrophage frequencies to females. Macrophages in the healthy mammary gland have been linked to the development and cyclic remodeling of the ductal network and the assistance of mammary stem cell function (Chua et al. 2010, Chakrabarti et al. 2018). The ductal network development has been studied in the CSF1 deficient *op/op* mice, which lack about 50% of $F4/80^{+}$ macrophages around the TEBs. (Gouon-Evans et al. 2000) The ductal development in *op/op* mice is delayed and somewhat perturbed in comparison to WT, but the network still fills the whole mammary gland and is able to produce milk. (Pollard, Henninghausen 1994) Unlike the development of fetal YS macrophages and adult bone marrow –derived macrophages, the development of fetal liver –derived macrophages is not CSF1R-dependent. It is possible that the fetal macrophages are still present in the *op/op* mammary gland and assist in the

ductal development. However, our data suggest that in addition to supporting the ductal remodeling and stem cell function, some of the mammary macrophages also serve an immunological role. We showed that the tissue-resident macrophages are superior in scavenging blood borne ligands, so it is likely that the tissue-resident macrophages are needed for the maintenance of immunological homeostasis of the mammaries in both sexes.

6.5 Future perspectives

6.5.1 PLVAP as a therapeutic target (I)

PLVAP is already the target of many researchers as its expression in the blood endothelium increases in many pathological conditions. We and others have shown that the PLVAP can be targeted and its function modulated by blocking antibodies. To evaluate the possible side effects of a new approach, knowledge of the expression pattern and kinetics of the target are beneficial. The discovery of PLVAP expression in the PLN, a major central for immune responses, could be useful for new ways to control inflammation. Excess activation and subsequent immune reactions by the PLN lymphocytes could potentially be regulated by targeted blocking of PLVAP in the SCS.

6.5.2 Mammary macrophages as a therapeutic target (III)

Macrophage presence in breast cancer has been mostly linked to poor prognosis, however their location in and around the tumor is clinically more relevant than their mere presence. Also, macrophages were not as abundantly present in all breast cancer types. (Medrek et al. 2012, Zhao et al. 2017) It is noteworthy that in other cancers, results on the macrophage effect on prognosis are both positive and negative. (Bingle et al. 2002) It seems that the macrophages in breast cancer (and other cancers) are heterogeneous in their phenotype and functions. Investigating the behavior of tissue-resident macrophages in breast cancer could produce new insight to the macrophage immunology.

It would be interesting to use the expression pattern to distinguish the embryonic macrophages ($F4/80^{Hi}CD64^{Hi}Siglec\text{-}1^{Hi}CD206^{Hi}$) in the mammary gland and investigate how they act in the development and progression of breast cancer. After carefully elucidating the functions of the resident macrophage populations, testing whether they could be modulated seems attractive. The detailed investigation of the similarities and differences of macrophage populations are warranted, since those factors are not only suitable druggable targets but also key points to avoid undesired adverse effects.

Macrophages can be used as carriers for drug delivery. (Klyachko et al. 2017) We established that the $CD206^{+}$ macrophages are superior in scavenging blood borne molecules. This functional ability that seems to separate the embryonic and adult macrophages in the mammary gland could potentially be utilized for the design of cell type specific, targeted therapies. Whether the therapy would be intended to eliminate the specific macrophages or to use them as carriers of medicine to the breast cancer, targeting the drug exposure could limit the systemic exposure to the drug, reduce the drug concentrations and thus limit adverse effects.

7 Conclusions

Collectively, the results in this thesis study show the decisive role of endothelium (and the protein PLVAP) in cell traffic and molecule exchange. We also show that the endothelium plays an important role in the formation of tissue-resident macrophage population in the mammary gland during embryogenesis. Disturbances, even transient ones, in the integrity of the endothelium can have serious and long lasting adverse effects in immune responses and immune cell composition of tissues.

The main conclusions of the study are the following

1. PLVAP is expressed in the lymphatic endothelium of the lymph node and regulates endothelial permeability in the sub capsular sinus and the activation of parenchymal lymphocytes.
2. In addition to forming physical sieve-like structures, PLVAP has the capability to participate in several molecular interactions.
3. PLVAP expression during embryogenesis is important for the emergence of F4/80^{Hi} tissue-resident macrophages in several tissues.
4. Mammary gland harbors a large macrophage population already before birth. In adulthood, F4/80^{Hi} macrophages constitute the major macrophage population in the mammary gland.
5. Tissue-resident macrophages perform unique scavenging functions in the mammary glands.

Acknowledgements

The work described in this thesis was carried out in the MediCity Research Laboratory, Institute of Biomedicine, University of Turku, Finland.

I wish to thank my supervisors Academician Sirpa Jalkanen, Professor Marko Salmi and Docent Pia Rantakari for providing me the opportunity to do my PhD here. You have showed me three outlooks on scientific research and the academic life. Sirpa, your enthusiasm and drive, Marko, your calm rationality and endless knowledge and Pia, your creativity and ambition have guided me through these years. The most important lesson I learned from you all is the passion for science.

Professor Risto Renkonen and Docent Marja Mikkola are warmly thanked for reviewing this thesis. Discussing the thesis with you was a pleasure and your comments and insight helped me improve this thesis.

Professor Ulla Pirvola, I thank you for being a part of my steering committee. Unfortunately we did not get together too often, but your input is still much appreciated.

The projects and publications in this thesis are products of intense collaboration. Therefore I thank all my co-authors for their efforts. With some we have worked side by side around the clock and shared the ups and downs of research life. PLVAP posse is thanked for the supportive and fun everyday work environment: Heidi Gerke, Emmi Lokka, Heli Jokela, Ines Felix, and Etta-Liisa Väänänen and the visiting students made sure that not only we got the job done, but we also had fun doing it. Fellow doctoral students Laura Kähäri and Ruth Fair-Mäkelä, thank you for the laughs and lively discussions about science and everything but science. I would also like to thank all the past and present people in the groups as well as the Maritta Pohjansalo, Riikka Sjöroos, Sari Mäki, Teija Kanasuo and Mari Parsama for their technical expertise. Jouko Sandholm, Ketlin Adel and Markku Saari, thank you for brightening my day each time we worked together, none of our projects would have been finished without you!

I thank Elina Wiik and Katri Kulmala for secretarial assistance and Antti-Pekka Laine for helping me when my IT skills weren't enough.

I am fortunate to have shared my journey to a PhD with so many friends. It has been wonderful to be able to discuss and get peer support and advice from my

friends (and fellow PhDs) Matias Knuuttila, Janne Hakkarainen, Juhani Aakko, and Sofia Aakko. I warmly thank the other friends from Terbio-years: Lauri Saastamoinen, Joona Valtonen, Jussi Jalonen, Tiia Asukas, Mia Ståhle, Riikka Siitonen and Mari Linhala. You have been a great peer support during this journey. Anna and the Lemberg family are thanked for the friendship and support over the years.

Kiitos äidilleni Mariannelle sekä Jarille, te olette kannustaneet minua jo ennen väitöskirjaurakkaani, mutta varsinkin sen aikana. Olette olleet ilahduttavan ylpeitä onnistumisistani ja myös tukeneet kun sen aika on ollut. Kiitokset myös veljilleni Jerrylle ja Lukelle, sekä Narikolle ja Yoritolle, muille sukulaisilleni, sekä Mallalle. Kiitos ”appivanhemmilleni” Marja-Tertulle ja Heikille mielenkiinnosta uraani kohtaan, kannustuksesta sen suhteen. Kiitos koko Sahan perheelle ja suvulle lämpimästä vastaanotosta joukkoonne.

Lopuksi, tahdon kiittää pientä perhettäni. Matti, ei ole itsestään selvää että löytää rinnalleen ihmisen, joka varauksetta kannustaa ja rakastaa. Sinä olet juuri sellainen minulle ja kanssasi on helppo olla onnellinen. Poikamme Ahto, olet mahtava ja rakas pieni ukkeli! Valaiset jokaisen päivän vilpittömällä hymylläsi ja toohotustasi on ilahduttava seurata. Odotan innolla tulevia kikkailujamme perheenä, kiitos että olette osa elämääni.

I want to acknowledge the TuDMM doctoral programme, Finnish Cultural Foundation – Satakunta Regional Fund, Turun Yliopistosäätio, Emil Aaltonen Foundation, Orion research Foundation, Instrumentarium Science foundation and Alfred Kordelin Foundation for financially supporting this thesis.

References

Aird WC (2012) Endothelial Cell Heterogeneity. Cold Spring Harbor perspectives in medicine, 2(1).

Ajami B, Bennet JL, Krieger C, Tetzlaff W, Rossi FMV (2007) Local self-renewal can sustain CNS microglia maintenance and function throughout adult life. Nature Neuroscience, 10(12), 1538-43.

Ålgars A, Karikoski M, Yegutkin GG, Stoitzner P, Niemelä J, Salmi M, Jalkanen S (2011) Different role of CD73 in leukocyte trafficking via blood and lymph vessels. Blood, 117(16), 4387-93.

Allan RS, Waithman J, Bedoui S, Jones CM, Villadangos JA, Zhan Y, Lew AM, Shortman K, Heath WR, Carbone FR (2006) Migratory Dendritic Cells Transfer Antigen to a Lymph Node-Resident Dendritic Cell Population for Efficient CTL Priming. Immunity, 25(1), 153-62.

Allende ML, Lesneski MJ, Xu Y, Brinkmann V, Cyster JG, Lo CG, Proia RL, Matloubian M, Cinamon G (2004) Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1. Nature, 427(6972), 355-60.

Anselmo AC, Mitragotri S (2017) Impact of particle elasticity on particle-based drug delivery systems. Advanced Drug Delivery Reviews, 108, 51-67.

Aspelund A, Antila S, Proulx ST, Karlsen TV, Karaman S, Detmar M, Wiig H, Alitalo K (2015) A dural lymphatic vascular system that drains brain interstitial fluid and macromolecules. Journal of Experimental Medicine, 212(7), 991-9.

Autran B, Carcelain G, Li TS, Blanc C, Mathez D, Tubiana R, Katlama C, Debre P, Leibowitch J (1997) Positive Effects of Combined Antiretroviral Therapy on CD4+ T Cell Homeostasis and Function in Advanced HIV Disease. Science, 277(5322), 112-6.

Bain CC, Bravo-Blas A, Scott CL, Perdiguero EG, Geissmann F, Henri S, Malissen B, Osborne LC, Artis D, Mowat AM (2014) Constant replenishment from circulating monocytes maintains the macrophage pool in adult intestine. Nature immunology, 15(10), 929-37.

Bajénoff M, Egen JG, Koo LY, Laugier J, Brau F, Glaichenhaus N, Germain RN (2006) Stromal Cell Networks Regulate Lymphocyte Entry, Migration, and Territoriality in Lymph Nodes. Immunity, 25(6), 989-1001.

Baluk P, McDonald DM (2008) Markers for Microscopic Imaging of Lymphangiogenesis and Angiogenesis. Annals of the New York Academy of Sciences, 1131(1), 1-12.

Baluk P, Fuxe J, Hashizume H, Romano T, Lashnits E, Butz S, Vestweber D, Corada M, Molendini C, Dejana E, McDonald DM (2007) Functionally specialized junctions between endothelial cells of lymphatic vessels. Journal of Experimental Medicine, 204(10), 2349-62.

Batista FD, Iber D, Neuberger MS (2001) B cells acquire antigen from target cells after synapse formation. Nature, 411(6836), 489-94.

Bearer EL, Orci L (1985) Endothelial fenestral diaphragms: a quick-freeze, deep-etch study. *The Journal of cell biology*, 100(2), 418-28.

Berney C, Herren S, Power CA, Gordon S, Martinez-Pomares L, Kosco-Vilbois MH (1999) A member of the dendritic cell family that enters B cell follicles and stimulates primary antibody responses identified by a mannose receptor fusion protein. *The Journal of experimental medicine*, 190(6), 851-60.

Bingle L, Brown NJ, Lewis CE (2002) The role of tumour-associated macrophages in tumour progression: implications for new anticancer therapies. *The Journal of Pathology*, 196(3), 254-65.

Bolander FF (1990) Differential characteristics of the thoracic and abdominal mammary glands from mice. *Experimental Cell Research*, 189(1), 142-4.

Bonapace L, Coissieux M, Wyckoff J, Mertz KD, Varga Z, Junt T, Bentires-Alj M (2014) Cessation of CCL2 inhibition accelerates breast cancer metastasis by promoting angiogenesis. *Nature.* 515, pages 130–133.

Boring L, Gosling J, Cleary M, Charo I (1998) Decreased lesion formation in CCR2$^{-/-}$ mice reveals a role forchemokines in the initiation of atherosclerosis. *Nature*, 894-7.

Bosma EK, van Noorden, Cornelis J. F, Schlingemann RO, Klaassen I (2018) The role of plasmalemma vesicle-associated protein in pathological breakdown of blood–brain and blood–retinal barriers: potential novel therapeutic target for cerebral edema and diabetic macular edema. *Fluids and Barriers of the CNS*, 15(1), 1-17.

Bovay E, Sabine A, Prat-Luri B, Kim S, Son K, Willrodt A, Olsson C, Halin C, Kiefer F, Betsholtz C, Jeon NL, Luther SA, Petrova TV (2018) Multiple roles of lymphatic vessels in peripheral lymph node development. *The Journal of experimental medicine*, 215(11), 2760-77.

Braun A, Worbs T, Moschovakis GL, Halle S, Hoffmann K, Bölter J, Münk A, Förster R (2011) Afferent lymph–derived T cells and DCs use different chemokine receptor CCR7–dependent routes for entry into the lymph node and intranodal migration. *Nature Immunology*, 12(9), 879-87.

Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A (2018) Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA: A Cancer Journal for Clinicians*, 68(6), 394-424.

Broekaert IJ, Becker K, Gottschalk I, Körber F, Dötsch J, Thiele H, Altmüller J, Nürnberg P, Hünseler C, Cirak S (2018) Mutations in plasmalemma vesicle-associated protein cause severe syndromic protein-losing enteropathy. *Journal of Medical Genetics*, 55(9), 637-40.

Brown M, Assen FP, Leithner A, Abe J, Schachner H, Asfour G, Bago-Horvath Z, Stein JV, Uhrin P, Sixt M, Kerjaschki D (2018) Lymph node blood vessels provide exit routes for metastatic tumor cell dissemination in mice. *Science*, 359(6382), 1408-11.

Buckley RH, Schiff SE, Schiff RI, Markert ML, Williams LW, Roberts JL, Myers LA, Ward FE (1999) Hematopoietic Stem-Cell Transplantation for the Treatment of Severe Combined Immunodeficiency. *The New England Journal of Medicine*, 340(7), 508-16.

Carman CV, Sage PT, Sciuto TE, de la Fuente, Miguel A., Geha RS, Ochs H, Dvorak HF, Dvorak AM, Springer TA (2007) Transcellular Diapedesis Is Initiated by Invasive Podosomes. *Immunity*, 26(6), 784-97.

Carrasco YR, Batista FD (2007) B Cells Acquire Particulate Antigen in a Macrophage-Rich Area at the Boundary between the Follicle and the Subcapsular Sinus of the Lymph Node. *Immunity*, 27(1), 160-71.

Chakarov S, Lim HY, Tan L, Lim SY, See P, Lum J, Zhang X, Foo S, Nakamizo S, Duan K, Kong WT, Gentek R, Balachander A, Carbajo D, Bleriot C, Malleret B, Tam JKC, Baig S, Shabeer M, Toh SES, Schlitzer A, Larbi A, Marichal T, Malissen B, Chen J, Poidinger M, Kabashima K, Bajenoff M, Ng LG, Angeli V, Ginhoux F (2019) Two distinct interstitial macrophage populations coexist across tissues in specific subtissular niches. *Science*, 363(6432), eaau0964.

Chi J, Chang H, Y, Haraldsen G, Jahnsen FL, Troyanskaya OG, Chang DS, Wang Z, Rockson SG, Van de Rijn M, Botstein D, O. Brown P (2003) Endothelial Cell Diversity Revealed by Global Expression Profiling. *Proceedings of the National Academy of Sciences of the United States of America*, 100(19), 10623-8.

Cho K, Morris D, DelProposto J, Geletka L, Zamarron B, Martinez-Santibanez G, Meyer K, Singer K, O'Rourke R, Lumeng C (2014) An MHC II-Dependent Activation Loop between Adipose

Tissue Macrophages and CD4+ T Cells Controls Obesity-Induced Inflammation. *Cell Reports*, 9(2), 605-17.

Chu EY, Hens J, Andl T, Kairo A, Yamaguchi TP, Brisken C, Glick A, Wysolmerski JJ, Millar SE (2004) Canonical WNT signaling promotes mammary placode development and is essential for initiation of mammary gland morphogenesis. *Development*, 131(19), 4819-29.

Chua ACL, Hodson LJ, Moldenhauer LM, Robertson SA, Ingman WV (2010) Dual roles for macrophages in ovarian cycle-associated development and remodelling of the mammary gland epithelium. *Development*, 137(24), 4229-38.

Clarke EL, Connell AJ, Six E, Kadry NA, Abbas AA, Hwang Y, Everett JK, Hofstaedter CE, Marsh R, Armant M, Kelsen J, Notarangelo LD, Collman RG, Hacein-Bey-Abina S, Kohn DB, Cavazzana M, Fischer A, Williams DA, Pai S, Bushman FD (2018) T cell dynamics and response of the microbiota after gene therapy to treat X-linked severe combined immunodeficiency. *Genome Medicine*, 10(1), 1-14.

Cleaver O, Krieg PA (2010) Chapter 8.2 - Vascular Development. *Heart Development and Regeneration*, 487-528.

Cohen JN, Guidi CJ, Tewalt EF, Qiao H, Rouhani SJ, Ruddell A, Farr AG, Tung KS, Engelhard VH (2010) Lymph node–resident lymphatic endothelial cells mediate peripheral tolerance via Aire-independent direct antigen presentation. *The Journal of experimental medicine*, 207(4), 681-8.

Couldrey C, Moitra J, Vinson C, Anver M, Nagashima K, Green J (2002) Adipose tissue: A vital in vivo role in mammary gland development but not differentiation. *Developmental Dynamics*, 223(4), 459-68.

Croxtall J, Van Hal P, Choudhury Q, Gilroy D, Flower R (2002) Different glucocorticoids vary in their genomic and non-genomic mechanism of action in A549 cells. *British Journal of Pharmacology*, 135(2), 511-9.

Dasari P, Sharkey DJ, Noordin E, Glynn DJ, Hodson LJ, Chin PY, Evdokiou A, Robertson SA, Ingman WV (2014) Hormonal regulation of the cytokine microenvironment in the mammary gland. *Journal of Reproductive Immunology*, 106, 58-66.

Davies LC, Rosas M, Smith PJ, Fraser DJ, Jones SA, Taylor PR (2011) A quantifiable proliferative burst of tissue macrophages restores homeostatic macrophage populations after acute inflammation. *European Journal of Immunology*.

Dick SA, Macklin J, A, Nejat S, Momen A, Clemente-Casares X, Althagafi MG, Chen J, Kantores C, Hosseinzadeh S, Aronoff L, Wong A, Zaman R, Barbu I, Besla R, Lavine KJ, Razani B, Ginhoux F, Husain M, Cybulsky MI, Robbins CS, Epelman S (2019) Self-renewing resident cardiac macrophages limit adverse remodeling following myocardial infarction. *Nature Immunology*, 20(1), 29-39.

Dini L, Lentini A, Diez GD, Rocha M, Falasca L, Serafino L, Vidal-Vanaclocha F (1995) Phagocytosis of apoptotic bodies by liver endothelial cells. *Journal of Cell Science*, 108(3), 967.

Duncan BB, Schmidt MI, Pankow JS, Ballantyne CM, Couper D, Vigo A, Hoogeveen R, Folsom AR, Heiss G (2003) Low-Grade Systemic Inflammation and the Development of Type 2 Diabetes: The Atherosclerosis Risk in Communities Study. *Diabetes*, 52(7), 1799-805.

Elgueta R, Tse D, Deharvengt SJ, Luciano MR, Carriere C, Noelle RJ, Stan RV (2016) Endothelial Plasmalemma Vesicle–Associated Protein Regulates the Homeostasis of Splenic Immature B Cells and B-1 B Cells. *Journal of Immunology*, 197(10), 3970-81.

Elkadri A, Thoeni C, Deharvengt SJ, Murchie R, Guo C, Stavropoulos JD, Marshall CR, Wales P, Bandsma RHJ, Cutz E, Roifman CM, Chitayat D, Avitzur Y, Stan RV, Muise AM (2015) Mutations in Plasmalemma Vesicle Associated Protein Result in Sieving Protein-Losing Enteropathy Characterized by Hypoproteinemia, Hypoalbuminemia, and Hypertriglyceridemia. *Cellular and Molecular Gastroenterology and Hepatology*, 1(4), 394.e7.

Ensan S, Li A, Besla R, Degousee N, Cosme J, Roufaiel M, Shikatani EA, El-Maklizi M, Williams JW, Robins L, Li C, Lewis B, Yun TJ, Lee JS, Wieghofer P, Khattar R, Farrokhi K, Byrne J, Ouzounian M, Zavitz CCJ, Levy GA, Bauer CMT, Libby P, Husain M, Swirski FK, Cheong C,

Prinz M, Hilgendorf I, Randolph GJ, Epelman S, Gramolini AO, Cybulsky MI, Rubin BB, Robbins CS (2015) Self-renewing resident arterial macrophages arise from embryonic CX3CR1+ precursors and circulating monocytes immediately after birth. *Nature Immunology,* 17(2),159-68

Escobedo N, Oliver G (2016) Lymphangiogenesis: Origin, Specification, and Cell Fate Determination. *Annual Review of Cell and Developmental Biology*, 32(1), 677-91.

Fata JE, Kong Y, Li J, Sasaki T, Irie-Sasaki J, Moorehead RA, Elliott R, Scully S, Voura EB, Lacey DL, Boyle WJ, Khokha R, Penninger JM (2000) The Osteoclast Differentiation Factor Osteoprotegerin-Ligand Is Essential for Mammary Gland Development. *Cell*, 103(1), 41-50.

Ferrara N, Carver-Moore K, Chen H, Dowd M, Lu L, O'Shea SK, Powell-Braxton L, Hillan KJ, Moore MW (1996) Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature*.

Fletcher AL, Malhotra D, Acton SE, Lukacs-Kornek V, Bellemare-Pelletier A, Curry M, Armant M, Turley SJ (2011) Reproducible Isolation of Lymph Node Stromal Cells Reveals Site-Dependent Differences in Fibroblastic Reticular Cells. *Frontiers in immunology*, 2, 35.

Forabosco P, Bouzigon E, Ng MY, Hermanowski J, Fisher SA, Criswell LA, Lewis CM (2009) Meta-analysis of genome-wide linkage studies across autoimmune diseases. *European Journal of Human Genetics*, 17(2), 236-43.

Förster C, Mäkelä S, Wärri A, Kietz S, Becker D, Hultenby K, Warner M, Gustafsson J (2002) Involvement of Estrogen Receptor β in Terminal Differentiation of Mammary Gland Epithelium. *Proceedings of the National Academy of Sciences of the United States of America*, 99(24), 15578-83.

Gautier EL, Shay T, Miller J, Greter M, Jakubzick C, Ivanov S, Helft J, Chow A, Elpek KG, Gordonov S, Mazloom AR, Ma'ayan A, Chua W, Hansen TH, Turley SJ, Merad M, Randolph GJ (2012) Gene-expression profiles and transcriptional regulatory pathways that underlie the identity and diversity of mouse tissue macrophages. *Nature Immunology*, 13(11), 1118.

Chakrabarti R, Celià-Terrassa T, Kumar S, Hang X, Wei Y, Choudhury A, Hwang J, Peng J, Nixon B, Grady JJ, DeCoste C, Gao J, van Es JH, Li MO, Aifantis I, Clevers H, Kang Y (2018) Notch ligand Dll1 mediates cross-talk between mammary stem cells and the macrophageal niche. *Science*, 360(6396), eaan4153.

Ginhoux F, Greter M, Leboeuf M, Nandi S, See P, Gokhan S, Mehler MF, Conway SJ, Guan Ng L, Stanley ER, Samokhvalov IM, Merad M (2010) Fate Mapping Analysis Reveals That Adult Microglia Derive from Primitive Macrophages. *Science,* 330(6005), 841-5.

Ginhoux F, Schultze JL, Murray PJ, Ochando J, Biswas SK (2016) New insights into the multidimensional concept of macrophage ontogeny, activation and function. *Nature Immunology*, 17(1), 34-40.

Ginhoux F, Guilliams M (2016) Tissue-Resident Macrophage Ontogeny and Homeostasis. *Immunity*, 44(3), 439-49.

Girard J, Moussion C, Förster R (2012) HEVs, lymphatics and homeostatic immune cell trafficking in lymph nodes. *Nature Reviews. Immunology*, 12(11), 762-73.

Gomez Perdiguero E, Klapproth K, Schulz C, Busch K, Azzoni E, Crozet L, Garner H, Trouillet C, de Bruijn MF, Geissmann F, Rodewald H (2015) Tissue-resident macrophages originate from yolk-sac-derived erythro-myeloid progenitors. *Nature*, 518(7540), 547-51.

Gordon EJ, Gale NW, Harvey NL (2008) Expression of the hyaluronan receptor LYVE-1 is not restricted to the lymphatic vasculature; LYVE-1 is also expressed on embryonic blood vessels. *Developmental Dynamics*, 237(7), 1901-9.

Gorelik L, Gilbride K, Dobles M, Kalled SL, Zandman D, Scott ML (2003) Normal B Cell Homeostasis Requires B Cell Activation Factor Production by Radiation-resistant Cells. *Journal of Experimental Medicine*, 198(6), 937-45.

Gouon-Evans V, Rothenberg ME, Pollard JW (2000) Postnatal mammary gland development requires macrophages and eosinophils. *Development*, 127(11), 2269.

Gouon-Evans V, Pollard JW (2002) Unexpected Deposition of Brown Fat in Mammary Gland During Postnatal Development. *Molecular Endocrinology*, 16(11), 2618-27.

Gretz JE, Norbury CC, Anderson AO, Proudfoot AE, Shaw S (2000) Lymph-borne chemokines and other low molecular weight molecules reach high endothelial venules via specialized conduits while a functional barrier limits access to the lymphocyte microenvironments in lymph node cortex. *The Journal of experimental medicine*, 192(10), 1425-40.

Grulich AE, van Leeuwen MT, Falster MO, Vajdic CM (2007) Incidence of cancers in people with HIV/AIDS compared with immunosuppressed transplant recipients: a meta-analysis. *Lancet*, 370(9581), 59-67.

Guilliams M, Kleer I, Henri S, Post S, Vanhoutte L, Prijck S, Deswarte K, Malissen B, Hammad H, Lambrecht B (2013) Alveolar macrophages develop from fetal monocytes that differentiate into long-lived cells in the first week of life via GM-CSF. *The Journal of Experimental Medicine*, 210(10), 1977-92.

Gyorki D, Lindeman G, Visvader J (2009) Resident Macrophages Influence Stem Cell Activity in the Mammary Gland. *Cancer Research*, 69(24 Supplement), 49.

Haldar M, Kohyama M, So A, KC W, Wu X, Briseño C, Satpathy A, Kretzer N, Arase H, Rajasekaran N, Wang L, Egawa T, Igarashi K, Baltimore D, Murphy T, Murphy K (2014) Heme-Mediated SPI-C Induction Promotes Monocyte Differentiation into Iron-Recycling Macrophages. *Cell*, 156(6), 1223-34.

Hammarlund E, Thomas A, Poore EA, Amanna IJ, Rynko AE, Mori M, Chen Z, Slifka MK (2016) Durability of Vaccine-Induced Immunity Against Tetanus and Diphtheria Toxins: A Cross-sectional Analysis. *Clinical Infectious Diseases*, 62(9), 1111-8.

Hashimoto D, Chow A, Noizat C, Teo P, Beasley M, Leboeuf M, Becker C, See P, Price J, Lucas D, Greter M, Mortha A, Boyer S, Forsberg E , Tanaka M, van Rooijen N, García-Sastre A, Stanley E , Ginhoux F, Frenette P, Merad M (2013) Tissue-Resident Macrophages Self-Maintain Locally throughout Adult Life with Minimal Contribution from Circulating Monocytes. *Immunity*, 38(4), 792-804.

Hattori K, Dias S, Heissig B, Hackett NR, Lyden D, Tateno M, Hicklin DJ, Zhu Z, Witte L, Crystal RG, Moore M, Rafii S (2001) Vascular Endothelial Growth Factor and Angiopoietin-1 Stimulate Postnatal Hematopoiesis by Recruitment of Vasculogenic and Hematopoietic Stem Cells. *Journal of Experimental Medicine*, 193(9), 1005-14.

Hennighausen L, Robinson GW (2001) Signaling Pathways in Mammary Gland Development. *Developmental Cell*, 1(4), 467-75.

Herrnberger L, Seitz R, Kuespert S, Bösl M, Fuchshofer R, Tamm E (2012) Lack of endothelial diaphragms in fenestrae and caveolae of mutant Plvap-deficient mice. *Histochemistry and Cell Biology*, 138(5), 709-24.

Hnasko R, McFarland M, Ben-Jonathan N (2002) Distribution and characterization of plasmalemma vesicle protein-1 in rat endocrine glands. *Journal of Endocrinology*, 175(3), 649-61.

Hoeffel G, Chen J, Lavin Y, Low D, Almeida F, See P, Beaudin A, Lum J, Low I, Forsberg E , Poidinger M, Zolezzi F, Larbi A, Ng L, Chan JY, Greter M, Becher B, Samokhvalov I, Merad M, Ginhoux F (2015) C-Myb+ Erythro-Myeloid Progenitor-Derived Fetal Monocytes Give Rise to Adult Tissue-Resident Macrophages. *Immunity*, 42(4), 665-78.

Hogg NAS, Harrison CJ, Tickle C (1983) Lumen formation in the developing mouse mammary gland. *Journal of Embryology and Experimental Morphology*, 73(1), 39.

Howard B, Gusterson B (2000) Human Breast Development. *Journal of Mammary Gland Biology and Neoplasia*, 5(2), 119-37.

Hu Z, Yang XY, Liu Y, Morse MA, Lyerly HK, Clay TM, Zhong P (2005) Release of endogenous danger signals from HIFU-treated tumor cells and their stimulatory effects on APCs. *Biochemical and Biophysical Research Communications*, 335(1), 124-31.

Ichikawa M, Sugita M, Takahashi M, Satomi M, Takeshita T, Araki T, Takahashi H (2003) Breast milk macrophages spontaneously produce granulocyte–macrophage colony-stimulating factor

and differentiate into dendritic cells in the presence of exogenous interleukin-4 alone. *Immunology*, 108(2), 189-95.

Ichimura K, Stan RV, Kurihara H, Sakai T (2008) Glomerular Endothelial Cells Form Diaphragms during Development and Pathologic Conditions. *Journal of the American Society of Nephrology: JASN*, 19(8), 1463-71.

Iftakhar-E-Khuda I, Fair-Mäkelä R, Kukkonen-Macchi A, Elima K, Karikoski M, Rantakari P, Miyasaka M, Salmi M, Jalkanen S (2016) Gene-expression profiling of different arms of lymphatic vasculature identifies candidates for manipulation of cell traffic. *Proceedings of the National Academy of Sciences of the United States of America*, 113(38), 10643-8.

Ingerman-Wojenski C, Silver MJ, Smith JB, Macarak E (1981) Bovine endothelial cells in culture produce thromboxane as well as prostacyclin. *The Journal of clinical investigation*, 67(5), 1292-6.

Ingman WV, Wyckoff J, Gouon-Evans V, Condeelis J, Pollard JW (2006) Macrophages promote collagen fibrillogenesis around terminal end buds of the developing mammary gland. *Developmental Dynamics*, 235(12), 3222-9.

Inman JL, Robertson C, Mott JD, Bissell MJ (2015) Mammary gland development: cell fate specification, stem cells and the microenvironment. *Development (Cambridge, England)*, 142(6), 1028-42.

Iwasaki A, Medzhitov R (2015) Control of adaptive immunity by the innate immune system. *Nature Immunology*, 16(4), 343-53.

Janeway CA (1992) The immune system evolved to discriminate infectious nonself from noninfectious self. *Immunology Today*, 13(1), 11-6.

Jerome-Majewska L, Jenkins GP, Ernstoff E, Zindy F, Sherr CJ, Papaioannou VE (2005) Tbx3, the ulnar-mammary syndrome gene, and Tbx2 interact in mammary gland development through a p19Arf/p53-independent pathway. *Developmental Dynamics*, 234(4), 922-33.

Jones D, Min W (2011) An overview of lymphatic vessels and their emerging role in cardiovascular disease. *Journal of cardiovascular disease research*, 2(3), 141-52.

Joshi PA, Jackson HW, Beristain AG, Di Grappa MA, Mote PA, Clarke CL, Stingl J, Waterhouse PD, Khokha R (2010) Progesterone induces adult mammary stem cell expansion. *Nature*, 465(7299), 803-7.

Jubinville É, Routhier J, Maranda-Robitaille M, Pineault M, Milad N, Talbot M, Beaulieu M, Aubin S, Paré M, Laplante M, Morissette MC (2019) Pharmacological activation of liver X receptor during cigarette smoke exposure adversely affects alveolar macrophages and pulmonary surfactant homeostasis. *American journal of physiology. Lung cellular and molecular physiology*, 316(4), L678.

Junt T, Moseman EA, Iannacone M, Massberg S, Lang PA, Boes M, Fink K, Henrickson SE, Shayakhmetov DM, Di Paolo NC, Van Rooijen N, Mempel TR, Whelan SP, Von Andrian UH (2007) Subcapsular sinus macrophages in lymph nodes clear lymph-borne viruses and present them to antiviral B cells. *Nature*.

Keuschnigg J, Henttinen T, Auvinen K, Karikoski M, Salmi M, Jalkanen S (2009) The prototype endothelial marker PAL-E is a leukocyte trafficking molecule. *Blood*, 114(2), 478-84.

Keuschnigg J, Tvorogov D, Elima K, Salmi M, Alitalo K, Salminen T, Jalkanen S (2012) PV-1 is recognized by the PAL-E antibody and forms complexes with NRP-1. *Blood*, 120(1), 232-5.

Klyachko NL, Polak R, Haney MJ, Zhao Y, Gomes Neto RJ, Hill MC, Kabanov AV, Cohen RE, Rubner MF, Batrakova EV (2017) Macrophages with cellular backpacks for targeted drug delivery to the brain. *Biomaterials*, 140, 79-87.

Koh TJ, DiPietro LA (2011) Inflammation and wound healing: the role of the macrophage. *Expert Reviews in Molecular Medicine*, 13, e23.

Kurolap A, Eshach-Adiv O, Gonzaga-Jauregui C, Dolnikov K, Mory A, Paperna T, Hershkovitz T, Overton JD, Kaplan M, Glaser F, Zohar Y, Shuldiner AR, Berger G, Baris HN (2018)

Establishing the role of PLVAP in protein-losing enteropathy: a homozygous missense variant leads to an attenuated phenotype. *Journal of medical genetics*, 55(11), 779-84.

Lambert BE, Moores SR (1986) Radiation Effects in the Lung. *Environmental Health Perspectives.*, 70, 261-91.

Lavin Y, Winter D, Blecher-Gonen R, David E, Keren-Shaul H, Merad M, Jung S, Amit I (2014) Tissue-Resident Macrophage Enhancer Landscapes Are Shaped by the Local Microenvironment. *Cell*, 159(6), 1312-26.

Lee SL, Wesselschmidt RL, Linette GP, Kanagawa O, Russell JH, Milbrandt J (1995) Unimpaired thymic and peripheral T cell death in mice lacking the nuclear receptor NGFI-B (Nur77). *Science*, 269(5223), 532-5.

Lerner A, Jeremias P, Matthias T (2016) The World Incidence and Prevalence of Autoimmune Diseases is Increasing. *International Journal of Celiac Disease*, 3(4), 151-5.

Lim HS, Blann AD, Chong AY, Freestone B, Lip GYH (2004) Plasma Vascular Endothelial Growth Factor, Angiopoietin-1, and Angiopoietin-2 in Diabetes: Implications for cardiovascular risk and effects of multifactorial intervention. *Diabetes care*, 27(12), 2918-24.

Link A, Vogt TK, Favre S, Britschgi MR, Acha-Orbea H, Hinz B, Cyster JG, Luther SA (2007) Fibroblastic reticular cells in lymph nodes regulate the homeostasis of naive T cells. *Nature Immunology*, 8(11), 1255-65.

Liu K, Victora G, Schwickert T, guermonprez P, Meredith M, Yao K, Chu F, Randolph G, Rudensky A, Nussenzweig M (2009) In Vivo Analysis of Dendritic Cell Development and Homeostasis. *Science*, 324, 392-7.

Loannidou S, Deinhardt K, Miotla J, Bradley J, Cheung E, Samuelsson S, Ng Y, Shima DT (2006) An in vitro Assay Reveals a Role for the Diaphragm Protein PV-1 in Endothelial Fenestra Morphogenesis. *Proceedings of the National Academy of Sciences of the United States of America*, 103(45), 16770-5.

Macias H, Hinck L (2012) Mammary gland development. *Wiley Interdisciplinary Reviews: Developmental Biology*, 1(4), 533-57.

Martinez FO, Gordon S (2014) The M1 and M2 paradigm of macrophage activation: time for reassessment. *F1000prime reports*, 6, 13.

Martinez-Corral I, Ulvmar M, Stanczuk L, Tatin F, Kizhatil K, John S, Alitalo K, Ortega S, Makinen T (2015) Nonvenous Origin of Dermal Lymphatic Vasculature. *Circulation Research*, 116(10), 1649-54.

Martínez-González J, Badimon L (2005) The NR4A subfamily of nuclear receptors: new early genes regulated by growth factors in vascular cells. *Cardiovascular research*, 65(3), 609-18.

Massberg S, Schaerli P, Knezevic-Maramica I, Köllnberger M, Tubo N, Moseman EA, Huff IV, Junt T, Wagers AJ, Mazo IB, von Andrian UH (2007) Immunosurveillance by Hematopoietic Progenitor Cells Trafficking through Blood, Lymph, and Peripheral Tissues. *Cell*, 131(5), 994-1008.

McGrath KE, Koniski AD, Malik J, Palis J (2003) Circulation is established in a stepwise pattern in the mammalian embryo. *Blood*, 101(5), 1669-75.

McHeyzer-Williams L, McHeyzer-Williams M (2005) Antigen- Specific Memory B cell Development. *Annual Review of Immunology*, 23(1), 487-513.

Medrek C, Pontén F, Jirström K, Leandersson K (2012) The presence of tumor associated macrophages in tumor stroma as a prognostic marker for breast cancer patients. *BMC Cancer*, 12(1), 306.

Melton DA, Cleaver O (2003) Endothelial signaling during development. *Nature Medicine*, 9(6), 661-8.

Mempel TR, Henrickson SE, Andrian U (2004) T-cell priming by dendritic cells in lymph nodes occurs in three distinct phases. *Nature*, 427(6970), 154-9.

Menconi F, Monti MC, Greenberg DA, Oashi T, Osman R, Davies TF, Ban Y, Jacobson EM, Concepcion ES, Li CW, Tomer Y (2008) Molecular Amino Acid Signatures in the MHC Class II

Peptide-Binding Pocket Predispose to Autoimmune Thyroiditis in Humans and in Mice. *Proceedings of the National Academy of Sciences of the United States of America*, 105(37), 14034-9.

Millán J, Hewlett L, Glyn M, Toomre D, Clark P, Ridley AJ (2006) Lymphocyte transcellular migration occurs through recruitment of endothelial ICAM-1 to caveola- and F-actin-rich domains. *Nature Cell Biology*, 8(2), 113-23.

Morris L, Graham CF, Gordon S (1991) Macrophages in haemopoietic and other tissues of the developing mouse detected by the monoclonal antibody F4/80. *Development*, 112(2), 517-26.

Mosca F, Tritto E, Muzzi A, Monaci E, Bagnoli F, Iavarone C, O'Hagan D, Rappuoli R, De Gregorio E (2008) Molecular and Cellular Signatures of Human Vaccine Adjuvants. *Proceedings of the National Academy of Sciences of the United States of America*, 105(30), 10501-6.

Mosser DM, Edwards JP (2008) Exploring the full spectrum of macrophage activation. *Nature Reviews*, 8.

Moussion C, Girard J (2011) Dendritic cells control lymphocyte entry to lymph nodes through high endothelial venules. *Nature*, 479(7374), 542-6.

M'Rini C, Cheng G, Schweitzer C, Cavanagh LL, Palframan RT, Mempel TR, Warnock RA, Lowe JB, Quackenbush EJ, von Andrian UH (2003) A Novel Endothelial L-Selectin Ligand Activity in Lymph Node Medulla That Is Regulated by α(1,3)-Fucosyltransferase-IV. *Journal of Experimental Medicine*, 198(9), 1301-12.

Mullican SE, Zhang S, Ruvolo V, Andreeff M, Conneely OM, Milbrandt J, Konopleva M (2007) Abrogation of nuclear receptors Nr4a3 and Nr4a1 leads to development of acute myeloid leukemia. *Nature Medicine*, 13(6), 730-5.

Nackiewicz D, Hanna RN, Hubbeling HG, Punt JA, Green AM, Hedrick CC, Geissmann F, Carlin LM (2011) The transcription factor NR4A1 (Nur77) controls bone marrow differentiation and the survival of Ly6C − monocytes. *Nature Immunology*, 12(8), 778-85.

Naik S, Bouladoux N, Wilhelm C, Molloy MJ, Salcedo R, Kastenmuller W, Deming C, Quinones M, Koo L, Conlan S, Spencer S, Hall JA, Dzutsev A, Kong H, Campbell DJ, Trinchieri G, Segre JA, Belkaid Y (2012) Compartmentalized Control of Skin Immunity by Resident Commensals. *Science*, 337(6098), 1115-9.

Nakagami Y, Hatano E, Chayama Y, Inoue T (2019) An anti-PLVAP antibody suppresses laser-induced choroidal neovascularization in monkeys. *European Journal of Pharmacology*, 854, 240-6.

Newton K, Dixit VM (2012) Signaling in Innate Immunity and Inflammation. *Cold Spring Harbor Perspectives in Biology*, 4(3), a006049.

Niemelä H, Elima K, Henttinen T, Irjala H, Salmi M, Jalkanen S (2005) Molecular identification of PAL-E, a widely used endothelial-cell marker. *Blood*, 106(10), 3405-9.

Nolte MA, Kraal G, Mebius RE (2004) Effects of fluorescent and nonfluorescent tracing methods on lymphocyte migration in vivo. *Cytometry. Part A*, 61(1), 35-44.

Nonaka H, Tanaka M, Suzuki K, Miyajima A (2007) Development of murine hepatic sinusoidal endothelial cells characterized by the expression of hyaluronan receptors. *Developmental dynamics : an official publication of the American Association of Anatomists*, 236(8), 2258-67.

Oakes SR, Hilton HN, Ormandy CJ (2006) Key stages in mammary gland development - The alveolar switch: coordinating the proliferative cues and cell fate decisions that drive the formation of lobuloalveoli from ductal epithelium. *Breas Cancer Research*, 8(2), 207.

Okabe Y, Medzhitov R (2014) Tissue-Specific Signals Control Reversible Program of Localization and Functional Polarization of Macrophages. *Cell*, 157(4), 832-44.

Palis J, Robertson S, Kennedy M, Wall C, Keller G (1999) Development of erythroid and myeloid progenitors in the yolk sac and embryo proper of the mouse. *Development*, 126(22), 5073.

Paolicelli RC, Bolasco G, Pagani F, Maggi L, Scianni M, Panzanelli P, Giustetto M, Alves Ferreira T, Guiducci E, Dumas L, Ragozzino D, Gross CT (2011) Synaptic Pruning by Microglia Is Necessary for Normal Brain Development. *Science*, 333(6048), 1456-8.

Park EJ, Lee JH, Yu G, He G, Ali SR, Holzer RG, Österreicher CH, Takahashi H, Karin M (2010) Dietary and Genetic Obesity Promote Liver Inflammation and Tumorigenesis by Enhancing IL-6 and TNF Expression. *Cell*, 140(2), 197-208.

Parmar H, Cunha GR (2004) Epithelial–stromal interactions in the mouse and human mammary gland in vivo. *Endocrine-Related Cancer*, 11(3), 437-58.

Pato E, Muñoz-Fernández S, Francisco F, Abad MA, Maese J, Ortiz A, Carmona L (2011) Systematic Review on the Effectiveness of Immunosuppressants and Biological Therapies in the Treatment of Autoimmune Posterior Uveitis. *Seminars in Arthritis and Rheumatism*, 40(4), 314-23.

Petrova TV, Koh GY (2018) Organ-specific lymphatic vasculature: From development to pathophysiology. *Journal of Experimental Medicine*, 215(1), 35-49.

Pollard JW, Henninghausen L (1994) Colony stimulating factor 1 is required for mammary gland development during pregnancy. *Proceedings of the National Academy of Sciences of the United States of America*, 91, 9312-6.

Predescu SA, Predescu DN, Palade GE (1997) Plasmalemmal vesicles function as transcytotic carriers for small proteins in the continuous endothelium. *American Journal of Physiology*, 41(2), H937.

Rafii S, Mohle R, Shapiro F, Frey BM, Moore MAS (1997) Regulation of Hematopoiesis by Microvascular Endothelium. *Leukemia & Lymphoma*, 27(5-6), 375-86.

Razani B, Engelman JA, Wang XB, Schubert W, Zhang XL, Marks CB, Macaluso F, Russell RG, Li M, Pestell RG, Di Vizio D, Hou HJ, Kneitz B, Lagaud G, Christ GJ, Edelmann W, Lisanti MP (2001) Caveolin-1 Null Mice Are Viable but Show Evidence of Hyperproliferative and Vascular Abnormalities. *Journal of Biological Chemistry*, 276(41), 38121.

Reynoso GV, Weisberg AS, Shannon JP, McManus DT, Shores L, Americo JL, Stan RV, Yewdell JW, Hickman HD (2019) Lymph node conduits transport virions for rapid T cell activation. *Nature immunology*.

Rhodin JAG (1962) The diaphragm of capillary endothelial fenestrations. *Journal of Ultrastructure Research*, 6(2), 171-85.

Ridker PM, Cushman M, Stampfer MJ, Tracy RP, Hennekens CH (1997) Inflammation, Aspirin, and the Risk of Cardiovascular Disease in Apparently Healthy Men. *The New England Journal of Medicine*, 336(14), 973-9.

Risau W, Sariola H, Zerwes HG, Sasse J, Ekblom P, Kemler R, Doetschman T (1988) Vasculogenesis and angiogenesis in embryonic-stem-cell-derived embryoid bodies. *Development,* 102(3), 471.

Risau W (1995) Differentiation of endothelium. *The FASEB Journal*, 9(10), 926-33.

Risau W, Flamme I (1995) Vasculogenesis. *Annual Review of Cell and Developmental Biology*, 11(1), 73-91.

Risau W (1998) Development and differentiation of endothelium. *Kidney International*, 54(S67), S6.

Roozendaal R, Mempel TR, Pitcher LA, Gonzalez SF, Verschoor A, Mebius RE, von Andrian UH, Carroll MC (2009) Conduits Mediate Transport of Low-Molecular-Weight Antigen to Lymph Node Follicles. *Immunity*, 30(2), 264-76.

Ryan GR (2001) Rescue of the colony-stimulating factor 1 (CSF-1)-nullizygous mouse (Csf1op/Csf1op) phenotype with a CSF-1 transgene and identification of sites of local CSF-1 synthesis. *Blood*, 98(1), 74-84.

Salomon B, Cohen JL, Masurier C, Klatzmann D (1998) Three Populations of Mouse Lymph Node Dendritic Cells with Different Origins and Dynamics. *The Journal of Immunology*, 160(2), 708-17.

Schlingemann RO, Dingjan GM, Emeis JJ, Blok J, Warnaar SO, Ruiter DJ (1985) Monoclonal antibody PAL-E Specific for endothelium. *Laboratory Investigation*.

Schubert W, Frank PG, Razani B, Park DS, Chow C, Lisanti MP (2001) Caveolae-deficient Endothelial Cells Show Defects in the Uptake and Transport of Albumin in Vivo. *Journal of Biological Chemistry*, 276(52), 48619.

Schulz C, Gomez Perdiguero E, Chorro L, Szabo-Rogers H, Cagnard N, Kierdorf K, Prinz M, Wu B, Jacobsen SEW, Pollard JW, Frampton J, Liu KJ, Geissmann F (2012) A Lineage of Myeloid Cells Independent of Myb and Hematopoietic Stem Cells. *Science*, 336(6077), 86-90.

Serbina NV, Pamer EG (2006) Monocyte emigration from bone marrow during bacterial infection requires signals mediated by chemokine receptor CCR2. *Nature Immunology*, 7(3), 311-7.

Shalaby F, Rossant J, Yamaguchi TP, Gertsenstein M, Wu X, Breitman ML, Schuh AC (1995) Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. *Nature*, 376(6535), 62-6.

Shetty S, Weston CJ, Oo YH, Westerlund N, Stamataki Z, Youster J, Hubscher SG, Salmi M, Jalkanen S, Lalor PF, Adams DH (2011) Common Lymphatic Endothelial and Vascular Endothelial Receptor-1 Mediates the Transmigration of Regulatory T Cells across Human Hepatic Sinusoidal Endothelium. *Journal of Immunology*, 186(7), 4147-55.

Shuvaev VV, Kiseleva RY, Arguiri E, Villa CH, Muro S, Christofidou-Solomidou M, Stan RV, Muzykantov VR (2018) Targeting superoxide dismutase to endothelial caveolae profoundly alleviates inflammation caused by endotoxin. *Journal of Controlled Release*, 272, 1-8.

Sica A, Mantovani A (2012) Macrophage plasticity and polarization: in vivo veritas. *The Journal of clinical investigation*, 122(3), 787-95.

Sindrilaru A, Peters T, Wieschalka S, Baican C, Baican A, Peter H, Hainzl A, Schatz S, Qi Y, Schlecht A, Weiss JM, Wlaschek M, Sunderkötter C, Scharffetter-Kochanek K (2011) An unrestrained proinflammatory M1 macrophage population induced by iron impairs wound healing in humans and mice. *The Journal of clinical investigation*, 121(3), 985-97.

Sixt M, Kanazawa N, Selg M, Samson T, Roos G, Reinhardt DP, Pabst R, Lutz MB, Sorokin L (2005) The Conduit System Transports Soluble Antigens from the Afferent Lymph to Resident Dendritic Cells in the T Cell Area of the Lymph Node. *Immunity*, 22(1), 19-29.

Soucie EL, Weng Z, Geirsdóttir L, Molawi K, Maurizio J, Fenouil R, Mossadegh-Keller N, Gimenez G, VanHille L, Beniazza M, Favret J, Berruyer C, Perrin P, Hacohen N, Andrau J, Ferrier P, Dubreuil P, Sidow A, Sieweke MH (2016) Lineage-specific enhancers activate self-renewal genes in macrophages and embryonic stem cells. *Science*, 351(6274), aad5510.

Stan RV, Ghitescu L, Jacobson BS, Palade GE (1999) Isolation, Cloning, and Localization of Rat PV-1, a Novel Endothelial Caveolar Protein. *The Journal of cell biology*, 1189-98.

Stan RV, Kubitza M, Palade GE (1999) PV-1 Is a Component of the Fenestral and Stomatal Diaphragms in Fenestrated Endothelia. *Proceedings of the National Academy of Sciences of the United States of America*, 96(23), 13203-7.

Stan RV, Arden KC, Palade GE (2001) cDNA and Protein Sequence, Genomic Organization, and Analysis of cis Regulatory Elements of Mouse and Human PLVAP Genes. *Genomics*, 72(3), 304-13.

Stan RV (2007) Endothelial stomatal and fenestral diaphragms in normal vessels and angiogenesis. *Journal of Cellular and Molecular Medicine*, 11(4), 621-43.

Stan RV, Tse D, Deharvengt S, Smits N, Xu Y, Luciano M, McGarry C, Buitendijk M, Nemani K, Elgueta R, Kobayashi T, Shipman S, Moodie K, Daghlian C, Ernst P, Lee H, Suriawinata A, Schned A, Longnecker D, Fiering S, Noelle R, Gimi B, Shworak N, Carrière C (2012) The Diaphragms of Fenestrated Endothelia: Gatekeepers of Vascular Permeability and Blood Composition. *Developmental Cell*, 23(6), 1203-18.

Stein JV, Soriano SF, M'rini C, Nombela-Arrieta C, de Buitrago GG, Rodríguez-Frade JM, Mellado M, Girard J, Martínez-A C (2003) CCR7-mediated physiological lymphocyte homing involves activation of a tyrosine kinase pathway. *Blood*, 101(1), 38-44.

Streeten EA, Brandi ML (1990) Biology of bone endothelial cells. *Bone and Mineral*, 10(2), 85-94.

Sternlicht MD (2006) Key stages in mammary gland development: The cues that regulate ductal branching morphogenesis. *Breast cancer research : BCR*, 8(1), 201.

Stolen CM, Marttila-Ichihara F, Koskinen K, Yegutkin GG, Turja R, Bono P, Skurnik M, Hänninen A, Jalkanen S, Salmi M (2005) Absence of the Endothelial Oxidase AOC3 Leads to Abnormal Leukocyte Traffic In Vivo. *Immunity*, 22(1), 105-15.

Strickland LA, Jubb AM, Hongo J, Zhong F, Burwick J, Fu L, Frantz GD, Koeppen H (2005) Plasmalemmal vesicle-associated protein (PLVAP) is expressed by tumour endothelium and is upregulated by vascular endothelial growth factor-A (VEGF). *The Journal of Pathology*, 206(4), 466-75.

Thierry GR, Kuka M, De Giovanni M, Mondor I, Brouilly N, Iannacone M, Bajénoff M (2018) The conduit system exports locally secreted IgM from lymph nodes. *The Journal of experimental medicine*, 215(12), 2972-83.

Thompson LF, Eltzschig HK, Ibla JC, Van De Wiele C, Justin, Resta R, Morote-Garcia JC, Colgan SP (2004) Crucial Role for Ecto-5 -Nucleotidase (CD73) in Vascular Leakage during Hypoxia, 200.

Tkachenko E, Tse D, Sideleva O, Deharvengt SJ, Luciano MR, Xu Y, McGarry CL, Chidlow J, Pilch PF, Sessa WC, Toomre DK, Stan RV (2012) Caveolae, Fenestrae and Transendothelial Channels Retain PV1 on the Surface of Endothelial Cells. *PLoS One*, 7(3), e32655.

Tsou C, Peters W, Si Y, Slaymaker S, Aslanian AM, Weisberg SP, Mack M, Charo IF (2007) Critical roles for CCR2 and MCP-3 in monocyte mobilization from bone marrow and recruitment to inflammatory sites. *Journal of Clinical Investigation*, 117(4), 902-9.

Umansky V, Sevko A (2012) Overcoming immunosuppression in the melanoma microenvironment induced by chronic inflammation. *Cancer Immunology, Immunotherapy*, 61(2), 275-82.

Van de Laar L, Saelens W, De Prijck S, Martens L, Scott C, Van Isterdael G, Hoffmann E, Beyaert R, Saeys Y, Lambrecht B, Guilliams M (2016) Yolk Sac Macrophages, Fetal Liver, and Adult Monocytes Can Colonize an Empty Niche and Develop into Functional Tissue-Resident Macrophages. *Immunity*, 44(4), 755-68.

Van Nguyen A, Pollard JW (2002) Colony Stimulating Factor-1 Is Required to Recruit Macrophages into the Mammary Gland to Facilitate Mammary Ductal Outgrowth. *Developmental Biology*, 247(1), 11-25.

Van Rooijen N, Sanders A (1994) Liposome mediated depletion of macrophages: mechanism of action, preparation of liposomes and applications. Journal of Immunological Methods, 174(1), 83-93

Varol C, Mildner A, Jung S (2015) Macrophages: Development and Tissue Specialization. *Annual Review of Immunology*, 33(1), 643-75.

Veerman K, Tardiveau C, Martins F, Coudert J, Girard J (2019) Single-Cell Analysis Reveals Heterogeneity of High Endothelial Venules and Different Regulation of Genes Controlling Lymphocyte Entry to Lymph Nodes. *Cell Reports*, 26(11), 3131.e5.

Veltmaat JM, Relaix F, Le LT, Kratochwil K, Sala FG, van Veelen W, Rice R, Spencer-Dene B, Mailleux AA, Rice DP, Thiery JP, Bellusci S (2006) Gli3-mediated somitic Fgf10 expression gradients are required for the induction and patterning of mammary epithelium along the embryonic axes. *Development*, 133(12), 2325.

Vestweber D (2015) How leukocytes cross the vascular endothelium. *Nature Reviews. Immunology*, 15(11), 692-704.

Wang Y, Cheng T, Chen T, Chang K, Chuang VP, Kao K (2014) Plasmalemmal Vesicle Associated Protein (PLVAP) as a therapeutic target for treatment of hepatocellular carcinoma. BMC Cancer, 14(1), 815.

Wang Y, Jin Y, Mäe M, Zhang Y, Ortsäter H, Betsholtz C, Mäkinen T, Jakobsson L (2017) Smooth muscle cell recruitment to lymphatic vessels requires PDGFB and impacts vessel size but not identity. *Development (Cambridge, England)*, 144(19), 3590.

Watson CJ, Khaled WT (2008) Mammary development in the embryo and adult: a journey of morphogenesis and commitment. *Development*, 135(6), 995-1003.

Wigle JT, Oliver G (1999) Prox1 Function Is Required for the Development of the Murine Lymphatic System. *Cell*, 98(6), 769-78.

Wilson JC, Sarsour K, Gale S, Pethö-Schramm A, Jick SS, Meier CR (2019) Incidence and Risk of Glucocorticoid-Associated Adverse Effects in Patients With Rheumatoid Arthritis. *Arthritis Care & Research*, 71(4), 498-511.

Wisniewska-Kruk J, van der Wijk A, van Veen HA, Gorgels, Theo G. M. F., Vogels IMC, Versteeg D, Van Noorden, Cornelis J. F., Schlingemann RO, Klaassen I (2016) Plasmalemma Vesicle–Associated Protein Has a Key Role in Blood-Retinal Barrier Loss. *The American Journal of Pathology*, 186(4), 1044-54.

Wolber FM, Leonard E, Michael S, Orschell-Traycoff CM, Yoder MC, Srour EF (2002) Roles of spleen and liver in development of the murine hematopoietic system. *Experimental Hematology*, 30(9), 1010-9.

Wood PJ (2015) Immunological response to infection: inflammatory and adaptive immune responses. *Anaesthesia & Intensive Care Medicine*, 16(7), 349-52.

Xu H, Guan H, Zu G, Bullard D, Hanson J, Slater M, Elmets CA (2001) The role of ICAM-1 molecule in the migration of Langerhans cells in the skin and regional lymph node. *European Journal of Immunology*, 31(10), 3085-93.

Yona S, Kim K, Wolf Y, Mildner A, Varol D, Breker M, Strauss-Ayali D, Viukov S, Guilliams M, Misharin A, Hume D, Perlman H, Malissen B, Zelzer E, Jung S (2013) Fate Mapping Reveals Origins and Dynamics of Monocytes and Tissue Macrophages under Homeostasis. *Immunity*, 38(1), 79-91.

Young AJ (1999) The physiology of lymphocyte migration through the single lymph node in vivo. *Seminars in Immunology*, 11(2), 73-83.

Zhao X, Qu J, Sun Y, Wang J, Liu X, Wang F, Zhang H, Wang W, Ma X, Gao X, Zhang S (2017) Prognostic significance of tumor-associated macrophages in breast cancer: a meta-analysis of the literature. *Oncotarget*, 8(18), 30576-86.

Zhu Y, Knolhoff BL, Meyer MA, Nywening TM, West BL, Luo J, Wang-Gillam A, Goedegebuure SP, Linehan DC, DeNardo DG (2014) CSF1/CSF1R Blockade Reprograms Tumor-Infiltrating Macrophages and Improves Response to T-cell Checkpoint Immunotherapy in Pancreatic Cancer Models. *Cancer research*, 74(18), 5057-69.

UNIVERSITY OF TURKU

Painosalama Oy, Turku , Finland 2019

ISBN 978-951-29-7780-2 (PRINT)
ISBN 978-951-29-7781-9 (PDF)
ISSN 0355-9483 (PRINT) ISSN 2343-3213 (ONLINE)