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PLVAP marks the exit

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ABSTRACT

Endothelium is the barrier between the blood and tissue stroma. Cells and macromolecules are selectively allowed to traffic through the endothelium to maintain homeostasis. Besides the paracellular transport route at the cell-cell junctions, transcellular pathways also exist. Fenestrae, transendothelial channels, and caveolae are specific structures of endothelial cells. They may be covered by a filtering unit called diaphragm, which is a proteinaceous structure that consists only of plasmalemma vesicle associated protein (PLVAP). PLVAP is known to participate in molecular sieving and cellular transmigration through the endothelium. Nevertheless, the role of PLVAP in the transendothelial cell migration has not been described during the foetal era.

The aims of this thesis were to study if PLVAP is functional in leukocyte trafficking in the foetal liver and to examine the dynamics of PLVAP expression in the liver sinusoidal endothelium. We found that PLVAP was needed for the efficient exit of foetal macrophage precursors from the liver to the bloodstream. Moreover, we discovered that the resident macrophage populations in adult mice were diminished under PLVAP deficiency. Surprisingly, we also observed nondiaphragmal PLVAP expression in the sinusoids of postnatal liver that persisted until adulthood. Finally, we aimed to study the resident macrophage ontogeny in depth in testis, which is known to accommodate a substantial macrophage population. Using the PLVAP-deficient mice and other models we revealed that testicular macrophages are mostly derived from foetal origins and that circulating monocytes in the adult mice have a negligible contribution to them. Furthermore, we showed that the resident macrophages in foetal testis are crucial for the normal spermatogenesis in mice.

These results are encouraging for further investigation of PLVAP functionalities in the context of general transendothelial leukocyte migration, but also for studying the functions of PLVAP outside diaphragms. Equally intriguing will be to further examine the functions of macrophages needed in the foetal testis to support normal tissue function.

KEYWORDS: Tissue development, tissue-resident macrophage, monocytes, testis, liver sinusoidal endothelium, haematopoiesis, PLVAP, transmigration

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TIIVISTELMÄ

Endoteeli muodostaa fyysisen esteen veren ja kudosten välille. Solujen ja makromolekyylien annetaan valikoiden läpäistä endoteeliseinämä kudosten homeostaasin ylläpitämiseksi. Endoteelisolujen välisten liitoskohtien lisäksi läpikulkureittejä on itse endoteelisoluissa. *Fenestrat* eli ikkunat, endoteelin läpäisevät kanavat sekä *caveolat* eli solukalvon rakkulamaiset sisentymät ovat endoteelisoluille ominaisia rakenteita. Niitä voi peittää suodattimen kaltainen diafragma-niminen rakenne, joka koostuu PLVAP (plasmalemma vesicle associated protein) proteiinista. PLVAP:in tiedetään osallistuvan molekyylien siivilöintiin sekä solujen siirtymiseen endoteelin läpi. PLVAP:in tehtävät sikiökaudella tunnetaan huonosti.

Tämän väitöskirjan tavoitteena oli selvittää, onko PLVAP:lla funktionaalista merkitystä alkion maksan valkosoluliikenteessä ja tutkia PLVAP:in ilmenemisen muutoksia maksan sinusoidien endoteelissä. Selvitimme, että PLVAP:ia tarvitaan makrofagien esiasteiden tehokkaaseen poistumiseen maksasta verenkiertoon. Saimme myös selville, että PLVAP-puutos johtaa pienentyneisiin kudosmakrofagipopulaatioihin. Havaitsimme yllättäen PLVAP ilmentymää maksan sinusoidien diafragmattomissa endoteelisoluissa syntymän jälkeen. Lopuksi pyrimme selvittämään syvemmin kudosmakrofagien alkuperää kiveksessä, jossa tiedetään olevan huomattava makrofagipopulaatio. Työssämme selvisi, että kiveksen makrofagit ovat enimmäkseen peräisin sikiökaudelta ja että vain pieni osa niistä tulee veren monosyyteistä. Lisäksi osoitimme, että alkion kiveksen kudosmakrofageja tarvitaan normaaliin hiiren siittiöiden kehitykseen.

Nämä tulokset rohkaisevat tutkimaan sekä PLVAP:in toiminnallisuutta laajemmin valkosolujen siirtymisessä endoteeliseinämien läpi että PLVAP:in toimintaa diafragmojen ulkopuolella. Yhtä kiinnostavaa olisi perehtyä syvemmin niihin makrofagien toimintoihin, joita alkion kiveksessä tarvitaan tukemaan kudoksen normaalia toimintaa.

AVAINSANAT: Kudoskehitys, kudosmakrofagi, monosyytit, kives, maksan sinusoidin endoteeli, hematopoieesi, PLVAP, transmigraatio

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Abbreviations

Adgre1	Adhesion G Protein-Coupled Receptor E1 (F4/80 antigen)
AMH	Anti-Müllerian hormone
BrdU	5-bromo-2´-deoxyuridine
BSA	Bovine serum albumin
BTB	Blood-testis-barrier
CCR2	C-C motif chemokine receptor 2
CD	Cluster of differentiation
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
CSF1	Colony stimulating factor 1
CSF1R	Colony stimulating factor 1 receptor
CSF2	Colony stimulating factor 2
CX3CR1	C-X3-C motif chemokine receptor 1
CyTOF	Cytometry by time-of-flight
DAMP	Damage-associated molecular pattern
DTA	Diphtheria toxin subunit A
DTR	Diphtheria toxin receptor
E	Embryonic day
ECL	Enhanced chemiluminescence
EMP	Erythro-myeloid progenitor
FACS	Fluorescence-activated cell sorting
FCS	Foetal calf serum
Flt3	FMS-related receptor tyrosine kinase 3
FSC	Forward scatter
GDNF	Glial cell line-derived neurotrophic factor
GMP	Granulocyte-monocyte-progenitor
HSC	Haematopoietic stem cell
HUVEC	Human umbilical vein endothelial cell
IL	Interleukin
i.p.	Intraperitoneal
i.v.	Intravenous

LDL	Low-density lipoprotein
LH	Luteinizing hormone
LMP	Lymphoid cell potential
LSEC	Liver sinusoidal endothelial cell
LTi	Lymphoid tissue inducer
LYVE-1	Lymphatic vessel endothelial hyaluronan receptor-1
Ly6C	Lymphocyte antigen 6 complex
MHC	Major histocompatibility complex
Ms4a3	Membrane-spanning 4-domains, subfamily A, member 3
NLR	Nucleotide oligomerization domain–like receptor
OCT	Optimal Cutting Temperature Compound
OVA	Ovalbumin
OVA-IC	Ovalbumin immune complex
P	Postnatal day
PAMP	Pathogen-associated molecular pattern
PAS	Periodic acid-Schiff
PECAM-1	Platelet and endothelial cell adhesion molecule 1
PLA	Proximity ligation assay
PLVAP	Plasmalemma vesicle-associated protein
PMC	Peritubular myoid cell
PPARγ	Peroxisome proliferator-activated receptor-gamma
RDH10	Retinol dehydrogenase 10
RT-qPCR	Quantitative real-time PCR
Runx1	Runt-related transcription factor 1
SDS	Sodium dodecyl sulphate
SEM	Scanning electron microscopy
Sry	Sex-determining region Y
Sry9	Sry box 9
SSC	Spermatogonial stem cell (or side scatter in flow cytometric plots)
TBS	Tris-buffered saline
TEC	Transendothelial channel
TEM	Transmission electron microscopy
TGFβ	Transforming growth factor beta
TLR	Toll-like receptor
TNF	Tumour necrosis factor
t-SNE	T-distributed Stochastic Neighbor Embedding
VEGF-A	Vascular endothelial growth factor A
VEGFR-2	Vascular endothelial growth factor receptor-2
Wnt	Wingless
WT	Wild type

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*, Norma Jäppinen*, Emmi Lokka, Elias Mokkala, Heidi Gerke, Emilia Peuhu, Johanna Ivaska, Kati Elima, Kaisa Auvinen & Marko Salmi. Foetal liver endothelium regulates the seeding of tissue-resident macrophages. *Nature*, 2016; 538: 392–396.

II Kaisa Auvinen, Emmi Lokka*, Elias Mokkala*, Norma Jäppinen, Sofia Tyystjärvi, Heikki Saine, Markus Peurla, Shishir Shetty, Kati Elima, Pia Rantakari & Marko Salmi. Fenestral diaphragms and PLVAP associations in liver sinusoidal endothelial cells are developmentally regulated. *Scientific reports*, 2019; 9: 15698.

III Emmi Lokka, Laura Lintukorpi, Sheyla Cisneros-Montalvo, Juho-Antti Mäkelä, Sofia Tyystjärvi, Venla Ojasalo, Heidi Gerke, Jorma Toppari, Pia Rantakari* & Marko Salmi*. Generation, localization and functions of macrophages during the development of testis. *Nature Communications*, 2020; 11: 4375.

(*authors contributed equally)

1 Introduction

The immune system is best known for its role in defending our bodies against microbes. Nevertheless, it is related to most, if not all, of the functions in the body. It is already involved in the early embryonic development where foetal macrophages participate in tissue remodelling. Another vital system already emerging in the foetal era is the vasculature. Endothelial cells are found in the inner surface of blood and lymphatic vessels where they maintain tissue homeostasis by regulating the trafficking of macromolecules, fluids, and adhesion, and the passage of immune cells. Plasmalemma vesicle-associated protein (PLVAP) is one of the molecules known to moderate these processes, especially through size-selective sieving of particles. Even though it has recently been shown to also regulate transendothelial cell migration, not much is known about this phenomenon. In this thesis I have investigated the role of PLVAP in the liver sinusoidal endothelial cells during foetal development, as well as in adulthood. Surprisingly, we found PLVAP to play a role in forming the resident macrophage populations that are constituted in the foetal era.

At birth, the body is introduced to a massive number of molecules, particles, and micro-organisms that are considered foreign but not necessarily dangerous. Hence, the immune system must be educated to recognize these agents as friends rather than enemies. After all, faulty immune reaction can be fatal. The first few days after the establishment of the immune cells are extremely important to build the tolerance of the immune system for self-antigens. However, in mammalian males, the first sperm emerges around the time of puberty expressing new self-antigens that would be considered foreign and therefore threatening. To prevent the immune system from attacking and thereby impairing sperm production, the testis environment must be tightly regulated. Resident macrophages represent one of the elements in maintaining immune privilege in the testis. In this thesis I have investigated the ontogeny and functions of the testicular macrophages. I discovered that they are derived mainly from the foetal monocytes and are needed for intact sperm production in mice.

Taken together, I have introduced PLVAP as a new molecule regulating the seeding of monocytes from foetal liver, I have shown that PLVAP expression persists in the liver sinusoidal endothelium in adulthood, and I have demonstrated

that the testicular macrophages are derived from embryonic sources and are essentially needed before birth to support normal spermatogenesis in mice.

2 Review of the Literature

2.1 Immune system

The main purpose of the immune system is to protect an organism against external threats and internal ones, when necessary. The immune system consists of multiple lines of defence provided by different leukocytes but also by other cell types. The first line to defend the body is the physical barrier formed by keratinized epithelium in the skin and the mucosa lining the cavities of the body. These structural barriers are accompanied by microbes residing on those surfaces, known as normal flora. Chemicals and enzymes can kill or digest microbes or toxins in the digestive tract. For instance, gastric juice containing hydrochloric acid in the stomach provides chemical clearing of most bacterial intruders that enter with food and drink. However, if the pathogens survive to pass these defence mechanisms and cause an infection, the cell-mediated immune response activates. This response can be divided into two main categories: innate and adaptive (i.e., acquired). The innate response is described as rapid and invariant, whereas the adaptive response is specified as delayed but tailored. (Turvey and Broide 2010) Even though the adaptive immune system has undeniable importance for the functions of the body in health and disease, this dissertation focuses on the development and functions of the innate immune system. Therefore, the innate immune system will be outlined in more detail in the next chapter.

2.1.1 Overview of innate immunity

Innate immunity consists of germline-encoded defence mechanisms acquired through the evolution of species. The cell types included in the innate immune system are the monocytes, macrophages, granulocytes, mast cells, megakaryocytes, dendritic cells, innate lymphocytes, and natural killer cells, as well as the epithelial cells. The physical barriers are considered to belong to the innate immune system as well. Indeed, one of the most essential tasks of the innate immune system is to prevent pathogens from entering the body or to fight them to maintain homeostasis. Nevertheless, during a pathogen challenge where intruders have proceeded through the barriers, the injured epithelial cells act as the first cellular responders. Epithelial

cells secrete signalling molecules that indicate the disruption of cell integrity and induce tissue inflammation. Such molecules are chemokines, cytokines, prostaglandins, and leukotrienes that can activate the leukocytes of the innate immune system. (Basset et al. 2003) In case of epithelial injury, there are also leukocytes residing in the lamina propria under the epithelium that can recognize the intruders and/or the signals from epithelial cells and activate. In general, resident leukocytes are found in all tissues where they conduct immune surveillance in a steady state. Finally, if the pathogens have direct access to the blood circulation as in the case of a cut wound, for instance, they are confronted by the circulating leukocytes, such as monocytes and neutrophils. (Iwasaki and Medzhitov 2015) Also, when passing the spleen vasculature, the resident immune cells help clear the pathogens from the blood. Therefore, splenectomised patients are at higher risk for sepsis after infection with *Streptococcus pneumonia*, for example. (Lewis, Williams, and Eisenbarth 2019) Additionally, there are antibodies and other proteins, such as those from the complement system or antimicrobial peptides, in the blood that help detect and fight the pathogens (Turvey and Broide 2010).

2.1.1.1 Innate immunity in health

The innate immune system has detection mechanisms for both external and internal threats. For the external ones, both epithelial cells and immune cells have pattern recognition receptors including Toll-like receptors (TLRs), nucleotide oligomerization domain–like receptors (NLRs), scavenger receptors, and complement receptors. They can identify pathogen-associated molecular patterns (PAMPs) such as macromolecules found on the surface of microbes or intracellular viral nucleic acids. Besides recognizing the pathogens, it is equally important to identify the healthy host cells. For example, natural killer cells receive inhibitory signals via major histocompatibility complex I (MHC I) expressed by uninfected and healthy host cells. Thus, infected or stressed cells with inadequate or missing self-signalling are targeted to be killed by the natural killer cells. (Iwasaki and Medzhitov 2015) Molecules that are released from injured or apoptotic cells are called damage-associated molecular patterns (DAMPs). They include endogenous alarmins and heat shock proteins that tag cells for clearance by immune cells. (Turvey and Broide 2010) In addition to these structural features, functional traits can be recognized. For example, multicellular parasites, that may lack structural patterns, are thought be identified through their functional features. Finally, the innate immune cells co-activate the adaptive immune system via antigen presentation and cytokine secretion. (Iwasaki and Medzhitov 2015)

Immunosuppression, in general, is also an important function of the innate immune system. For example, in the digestive tract, where a massive number of new

antigens are introduced to the body and where the commensal flora is enormous, immune surveillance must detect pathogens while leaving the nutrients and beneficial bacteria alone. This balance is partly regulated by macrophages, dendritic cells and innate lymphoid cells in the gut, where they secrete cytokines promoting or suppressing the immune response. (Kayama and Takeda 2016) The innate immune cells also play a central role in tissue regeneration and repair. Resident macrophages, especially, are known to take part in tissue maintenance by phagocytosing apoptotic cells or inducing cell proliferation by producing growth factors (Hirayama, Iida, and Nakase 2017).

2.1.1.2 Innate immunity in disease

The innate immune system is not only a friend but also a foe, even unintentionally. Immunodeficiency, related to poor function of immune cells or just the lack of immune cells due to genetic or independent external causes, is an apparent threat to an organism if any of the functions described in the previous chapter are impaired (Turvey and Broide 2010). Chronic inflammation, where an intervention to resolve the inflammation has failed, can lead to some pathologies. For instance in atherosclerosis, innate immune cells, specifically macrophages and monocytes, are out of balance due to high LDL concentration in the blood which eventually leads to the formation of foam cells, atherosclerotic plaques, and prolonged inflammation (Tall and Yvan-Charvet 2015). The innate immune system may also cause harm to the host while attempting to abolish pathogens. SARS-CoV-2 is one of the recent reminders of how the immune system can cause severe collateral damage. In some patients the innate immune cells, namely neutrophils, natural killer cells, and macrophages, are involved in the systemic inflammatory syndrome called cytokine storm which can lead to fatal multiorgan failure (Fajgenbaum and June 2020). With cancer, the cancer cells have the ability to escape the immune system, and they can turn innate immune cells from antitumour to protumour phenotype. For example, macrophages and neutrophils are known to be recruitable by cancer cells. (Maiorino et al. 2022)

2.1.1.3 Leukocytes of the innate immune system

In general, leukocytes that do not retain the memory of pathogen encounters are considered to represent the innate immune cells. These cells are the first responders to pathogen attacks alongside the physical and physiological barriers that keep invaders out of the system. As innate immune cells do not possess clonal memory from their encounters, and their response mechanisms are hard coded in their

genome, they always perform their manoeuvres in the same way. Both major immune cell lineages, lymphoid and myeloid, contribute to innate immunity.

The lymphoid cell lineage is better known for its repertoire of cells included in adaptive immunity. However, during the last decade, innate lymphoid cells have been acknowledged. These cells are practically innate counterparts for the different subtypes of T cells. Innate lymphoid cells have been divided into five subtypes: natural killer cells, ILC1-3, and lymphoid tissue inducers (LTi). They reside in tissues, lack antigen specificity, and react quickly to a wide variety of intra- and extracellular threats mainly by releasing cytokines which lure other immune cells to the site. (Vivier et al. 2018; Nagasawa, Spits, and Ros 2018)

Myeloid cell lineage produces erythrocytes, platelet-producing megakaryocytes, and different leukocyte types, all of which belong to the innate immune system. Granulocytes, named for their highly granular cytoplasm, include neutrophils, basophils, and eosinophils. They can be identified individually by the contents and appearance of their granules and the distinct multilobular morphologies of their nuclei. Granulocytes protect individuals against different pathogens by releasing their granular contents to the extracellular space by exocytosis or even by cytolysis. Granules may contain cytotoxic compounds that kill the target or other molecules that support the actions of other immune cells, such as cytokines or other effector molecules. Granulocytes are also known to have important roles in tissue development, homeostasis, and repair. (Burn et al. 2021; McBrien and Menzies-Gow 2017; Miyake et al. 2021) Mast cells represent another highly granular cell type. They are tissue-resident leukocytes that are often found within the mucosal surfaces. Mast cells contribute to innate immunity, for example, by providing components for regulating vascular permeability and for promoting other effector cell actions. (Varricchi et al. 2019)

Monocytes represent the largest immune cell type in the blood, with a nucleus shaped like a horseshoe or a kidney. They can be classified into subtypes by their surface marker expression and functions in both humans and mice. The monocytes expressing lymphocyte antigen 6 complex (Ly6C) and C-C motif chemokine receptor 2 (CCR2) in mice and cluster of differentiation 14 (CD14) in humans are called classical or inflammatory monocytes. (Geissmann, Jung, and Littman 2003) The latter name refers to the ability of these cells to infiltrate inflamed tissue in a manner that is highly dependent on the CCR2. In inflamed tissues, monocytes can mature into effector cells, i.e., macrophages or dendritic cells. In a steady state, $Ly6C^{high}$ monocytes are circulating for approximately 24 hours, after which they may migrate into tissues and develop into resident macrophages, which will be described in more detail later, or they may mature into another monocyte subtype that is described below. (Guilliams, Mildner, and Yona 2018)

In mice, the subpopulation of monocytes lacking Ly6C and CCR2 expression but having upregulated C-X3-C motif chemokine receptor 1 (CX3CR1) is called nonclasscial or patrolling monocytes. Human equivalents of these monocytes co-express CX3CR1 and CD16 and are $CD14^{low}$. (Geissmann, Jung, and Littman 2003) $Ly6C^{low/-}$ monocytes are shown to derive from $Ly6C^{high}$ monocytes both in blood and bone marrow (Yona et al. 2013). These monocytes are called the patrolling monocytes as they circulate twice as long as classical monocytes (Yona et al. 2013), and instead of just floating freely in the blood, they crawl along the endothelial wall of the vessels (Auffray et al. 2007). In the vascular wall, they have tasks related to the maintenance of endothelium integrity, including the removal of apoptotic endothelial cells (Carlin et al. 2013). During inflammation, $Ly6C^{low/-}$ monocytes secrete tumour necrosis factor (TNF) which activates neutrophils, for example. In addition to the aforementioned monocyte subsets, a third one with intermediate Ly6C expression has been identified in mice. It is a heterogeneous population including cells that are suggested to give rise to monocyte-derived dendritic cells. A similar heterogeneous population of $CD14^{+}$ $CD16^{+}$ cells has been identified in humans. (Guilliams, Mildner, and Yona 2018)

2.1.2 Origins of the leukocytes of innate immunity

The Greek-originated word haematopoiesis refers to the process of making blood. In this step-by-step process, haematopoietic stem cells (HSCs) give rise to all of the cells found in the blood. Haematopoiesis is initiated in the early foetal period and continues until the end of life. Only the site of the action changes through aging.

2.1.2.1 Anatomy of the haematopoietic sites

Haematopoiesis and the generation of vasculature are launched in the early developmental stages. In mice, the first blood cells, including the primitive macrophage and megakaryocyte/erythroid progenitors, emerge in the blood islands of the extra embryonic yolk sac around embryonic day 7.0 (E7.0). Within two days, the primitive erythroid progenitors disappear altogether, highlighting the transient nature of this primitive wave of haematopoiesis. The second, transient definitive, wave starts soon after E8.0, just prior to the establishment of circulation. The haemogenic endothelium of the yolk sac gives rise to erythro-myeloid progenitors (EMP) that can differentiate into erythroid and myeloid lineage cells. (Palis et al. 1999; Tober et al. 2007)

Concurrently with EMP production at E8.5 in the yolk sac, the haemogenic endothelium in the para-aortic splanchnopleura of a mouse embryo gives rise to the first immature HSCs (Godin, Dieterlen-Lièvre, and Cumano 1995). In addition,

similar potential for HSC production has been suggested for the umbilical and vitelline arteries and placenta (Gekas et al. 2005; Ottersbach and Dzierzak 2005). HSC precursors mature into their full potency and reach peak numbers as the embryonic mesoderm further evolves to the aorta-gonad-mesonephros (AGM) region at E10.5. HSCs found in the vascular beds of the AGM region have been shown to possess multilineage potential, including lymphoid potential, and the ability to colonize adult bone marrow. (Müller et al. 1994; Medvinsky and Dzierzak 1996)

Haematopoietic progenitors from the yolk sac, AGM, and likely the placenta start to colonize the liver rudiment around E10.5. The foetal liver does not produce any haematopoietic progenitors itself but provides a favourable environment for the colonizing cells to expand and differentiate. (Kumaravelu et al. 2002) Around E11.5-12.5 it starts to deliver different leukocytes to circulation and peripheral tissues at varying developmental stages (McGrath et al. 2015). These include also long-term HSCs (LT-HSCs). The LT-HSCs have been shown to colonize the spleen starting from E14.5. The formation of vascularized bone marrow around E16.5 potentiates its colonization by HSCs. However, HSCs isolated from foetal bone marrow were able to successfully reconstitute the irradiated adult bone marrow niche only after E17.5. (Christensen et al. 2004)

After birth, the dramatic changes in the blood flow dynamics in the foetal liver lead to the gradual loss of the HSC niche. Hence, bone marrow gradually takes over the duties as the main haematopoietic source and continues that function for the rest of a lifetime. (Khan et al. 2016) Notably, the spleen serves as an emergency source for leukocytes that it can readily produce from the HSCs residing in the perivascular niche in the red pulp area (Short et al. 2019). The same haematopoietic sites described in mice have been identified in humans as well (Ivanovs et al. 2017).

2.1.2.2 From foetal to adult haematopoiesis

Primitive macrophages, erythroid cells, and megakaryocytes are produced in the primitive wave of haematopoiesis in the yolk sac. Recent findings have suggested that these cells would have a dual background from precursors called haemangioblast and haematomesoblast that are differentiated from the mesoderm and have the additional ability to differentiate into endothelium or mesenchyme, respectively (Biben et al. 2023). For example, microglia, the resident macrophage population in the brain, are largely derived from the primitive wave of haematopoiesis and therefore likely have originated from the haemangioblast and haematomesoblast (Ginhoux et al. 2010).

EMPs produced in the transient definitive wave derive from the haemogenic endothelium in the yolk sac through an endothelial-to-haematopoietic transition

which has been shown to be dependent on the *Runx1* (Runt-related transcription factor 1) (Chen et al. 2009). EMPs have the capacity to produce erythroid cells and a wide repertoire of myeloid cells, including monocytes and macrophages (McGrath et al. 2015). Of note, later but prior to HSC emergence, haematopoietic progenitors with shared lymphoid and myeloid cell potential (LMP) also appear from the yolk sac (Böiers et al. 2013). Although HSCs rise simultaneously with EMPs, the erythropoiesis has been shown to rely heavily on the latter until birth (Soares-Da-Silva et al. 2021).

Definitive HSCs are the shared haematopoietic cells between embryos and adults. They first emerge from the arterial compartments of the embryo as precursors. After differentiation to mature HSCs, with distinct expression profiles of Lin^{-}, Kit^{+} and $Sca1^{+}$, they expand in the foetal liver and start to colonize the foetal spleen and bone marrow. (Christensen et al. 2004) The debate about whether the HSCs contribute to haematopoiesis in the perinatal era has continued until recently. The discovery of a lineage tracing model based on labelling of the *Mds1*$^{+}$ haemogenic endothelium at E9.5 enabled researchers to track HSCs specifically and to show that HSCs were producing differentiated progeny in foetal liver (Zhang et al. 2021). However, others have shown that embryonic haematopoiesis is conducted independently of HSCs by using lineage tracing based on *Evi1*, which is highly expressed in both foetal and adult HSCs. Furthermore, it is suggested that the arterial-derived progenitors, also expanded and differentiated in the foetal liver, would contribute alongside the EMPs to prenatal haematopoiesis. (Yokomizo et al. 2022; Goyama et al. 2008)

When HSCs colonize the foetal bone marrow, they quickly adapt to the quiescent phenotype seen in the adult bone marrow. They are then self-renewed through slow but constant proliferation. One cell out of over 100 LT-HSCs is differentiated to short-term HSC, which lacks the long-term self-renewal ability, but seems to proliferate at a remarkably higher rate and therefore be responsible for the supply of differentiated leukocytes in the bone marrow and blood. (Busch et al. 2015) Interestingly, some leukocyte types, such as tissue-resident macrophages and certain lymphoid lineages, are renewed with minor or no contribution from HSC-derived haematopoiesis (Sawai et al. 2016). A summary of the different haematopoietic organs and the different haematopoietic progenitors is illustrated in **Figure 1**.

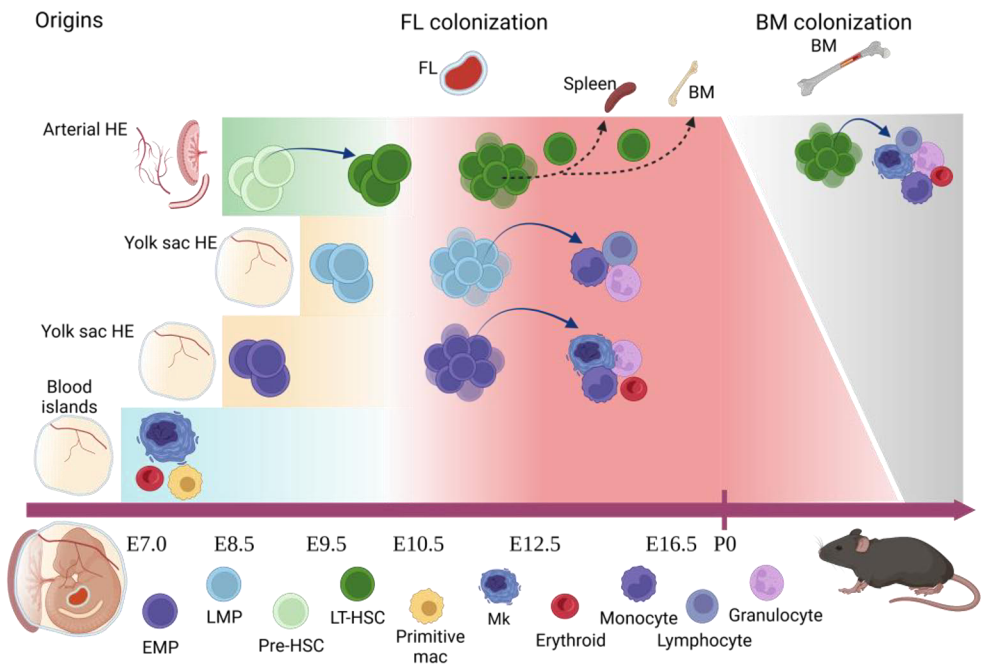

Figure 1. A schematic summary of the haematopoietic organs and progenitors at different time points. The first primitive haematopoiesis starts in the blood islands of the yolk sac generating a limited amount of leukocyte lineages, including primitive macrophages (primitive mac) and megakaryocytes (mk). Then haemogenic endothelium (HE) in the yolk sac and in the placenta, and vitelline artery and para-aortic splanchnopleura produce EMPs and HSC precursors (pre-HSC). Briefly after, LMPs emerge from the haemogenic endothelium in the yolk sac. Meanwhile, pre-HSCs have matured to long-term HSCs (LT-HSC) in the embryo. Around E10.5 the EMPs, LMPs and HSCs colonize the foetal liver (FL) where they expand in numbers and start to produce progeny. EMPs produce erythro-myeloid lineages while LMPs produce progenies from lympho-myeloid lineages. HSCs may contribute to this foetal liver haematopoiesis as well. However, the HSCs begin to colonize the spleen and bone marrow (BM) before birth. After birth haematopoiesis gradually shifts from liver to BM, which produces progenies of all leukocyte lineages. Created with BioRender.com.

2.1.3 Blood vascular endothelium

The vascular endothelium forms the innermost layer in the blood vessels. It is lying on top of the basal lamina and together these comprise the vascular intima. The endothelium is in direct contact with the blood content and therefore is in a key position to regulate the transfer of macromolecules, cells, and fluids between vasculature and tissues. It is exposed to mechanical stress caused by blood flow,

termed shear stress, as well as stretch caused by the physical changes in the environment, such as dilation of the alveoli in the lungs. (Krüger-Genge et al. 2019)

Endothelial cells, which are the endothelium formatting cells, differentiate from the mesodermal-originated angioblasts around E7.0 (Naito, Iba, and Takakura 2020). The same precursors produce primitive haematopoietic cells in the blood islands of the yolk sac (Biben et al. 2023). The process where the angioblasts in the borders of the blood islands differentiate into endothelial cells and start to build a primitive vascular network is called vasculogenesis. Simultaneously and similarly, the angioblasts in the embryo itself produce the dorsal aorta and cardinal vein. After the establishment of the first vessels, they continue to grow through sprouting or splitting. This process is called angiogenesis, and it is mediated by the proliferation and migration of the endothelial cells according to angiogenic stimuli, such as hypoxia, from the surrounding environment. (Naito, Iba, and Takakura 2020)

Vascular endothelium can be roughly categorized into three main types: continuous, fenestrated and discontinuous, which are illustrated in **Figure 2**. Continuous endothelium represents the most general type of endothelium found in the blood vessels with restricted permeability. The cells of the continuous endothelium are connected to each other by tight junctions and are anchored to the continuous basement membrane. Most of the peripheral vessels and the capillaries in the brain are lined by the continuous endothelium. In fenestrated endothelium, small transcellular pores are found in the periphery of the endothelial cells. Diaphragms can cover these pores, or they may remain open. Fenestrated diaphragmal endothelium is present in the hormone-secreting glands and kidney, for instance. The discontinuous endothelium with gap junctions and open fenestrae lines the special capillaries called sinusoids found in the spleen, liver, and bone marrow. (Krüger-Genge et al. 2019)

2.1.3.1 Endothelial cells

Endothelial cells typically lie flat in the interior of vessels. They are thin cells with luminal to abluminal polarization. They are anchored to the self-made, glycoprotein-constituted, basal lamina and have a negatively charged glycocalyx of varying thickness covering their luminal surface. In a steady state, endothelial cells are long-lived and described as quiescent. However, as an exception, in the uterus and corpus luteum, they proliferate in a cyclical manner. Endothelial cells show high heterogeneity, not only between different vessel types and different organs, but also within a vascular bed. (Aird 2007; Krüger-Genge et al. 2019) Numerous growth factors, adhesion and signalling molecules, along with oxygen and the mechanical stress from blood flow, regulate the actions of the endothelial cells (Ribatti 2006).

While fluid and small solutes can travel through the endothelium in the intercellular space, the endothelial cells are also known to be involved in the transcellular trafficking of macromolecules. Endocytosis is mediated via clathrin-coated pits in which macromolecules are encapsulated, internalized, and either recycled or destroyed by fusion with lysosomes. This process sometimes involves activation of the scavenger receptors. Transcytosis is a form of endocytosis where macromolecules are transported through the endothelial cells to be released in the opposing space. (Aird 2007) Certain cargo, such as albumin, is transported via transcytosis in plasmalemmal vesicles called caveolae (Schubert et al. 2001). The caveolae are flask-like invaginations of the plasma membrane and are constituted among other proteins by caveolin-1 (Aird 2007). Besides transcytosis, signal transduction, lipid homeostasis, and acting as excess membrane storage to protect the endothelial cells in case of increased mechanical stress have also been proposed as functions for the caveolae (Cheng and Nichols 2016). Other endothelial cell-specific structures are the fenestrae and transendothelial channels (TEC) through which macromolecules can diffuse. Fenestrae are pore openings found in the periphery of certain endothelial cells. In some tissues and regions, they can be grouped together into specific clusters called sieve plates. TEC are like fenestrae, but while the fenestrae can be described as pore-like structures the TEC are more like open-ended tunnels. (Aird 2007)

Describing the endothelial cell heterogeneity in the blood vasculature, let alone the lymphatic vasculature, in detail could be a subject for another thesis. However, as the sinusoidal capillaries in the liver play an essential role in the studies presented in this thesis, the next section will concentrate on describing the liver sinusoidal endothelial cells (LSEC).

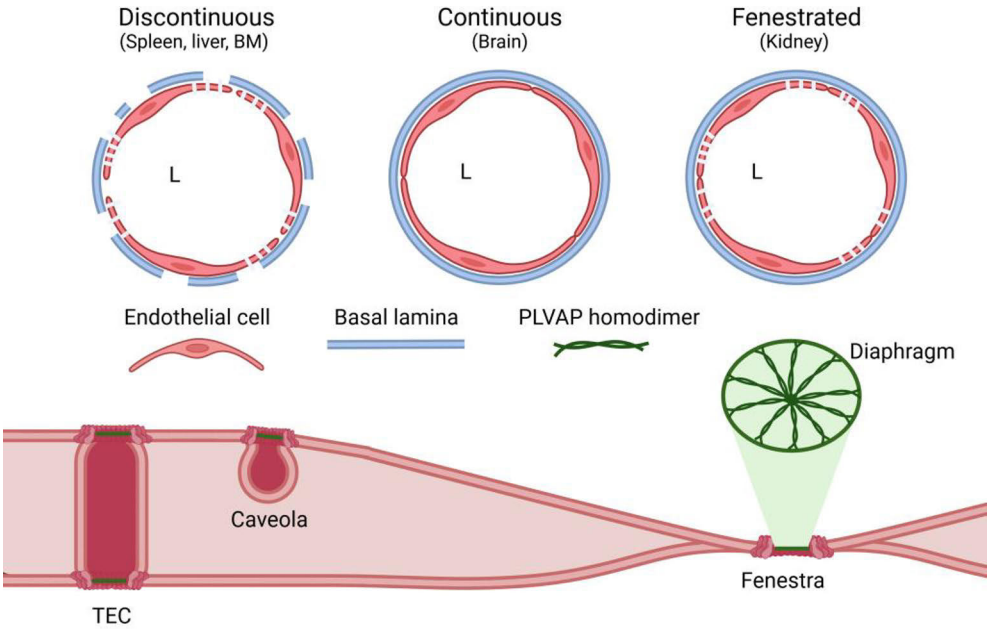

Figure 2. A schematic presentation of different endothelium types and endothelial cell structures. Shown are discontinuous, continuous, and fenestrated endothelia. Examples of tissues where each type can be found are indicated within parentheses. Below them is the representation of the transendothelial channel (TEC), caveola, and fenestra in the thinning of the endothelial cell periphery. The cartwheel-like diaphragm is formed by PLVAP and is illustrated too. Lumen, L, bone marrow, BM. Created with BioRender.com.

2.1.3.2 Liver sinusoidal endothelial cells (LSEC)

Endothelial cells play a central role in the morphogenesis of the liver as they guide the cells from the endoderm to form the liver bud. The LSEC precursors are observed since E9.0, and within a day, they have grown the first sinusoids induced by vascular endothelial growth factor receptor-2 (VEGFR-2) activation by its ligand, VEGF-A. (Matsumoto et al. 2001) The differentiation from continuous to discontinuous endothelium is essential for the haematopoietic cells to be able to colonize and therefore to proliferate and differentiate in the foetal liver. The differentiation is suggested to be orchestrated by the GATA4 transcription factor and can be detected as a loss of platelet and endothelial cell adhesion molecule 1 (PECAM-1 or CD31), highly expressed in continuous endothelium, and as an upregulation of lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1) and stabilin-2. (Géraud et al. 2017) Portal vessels branch in a fractal manner and thus provide a growing amount of niches for the proliferation and maintenance of the HSCs. The portal vessels

display arterial phenotype in the foetal era when they drain blood from the umbilical vein and distribute it through central vessels providing oxygen and nutrients for other foetal tissues. When dynamics in the blood circulation change after birth, the portal vein loses the artery features and the HSCs start to disappear from their perivascular niche in the liver. (Khan et al. 2016)

The liver consists of anatomically and physiologically distinct units called lobules which can be further divided into zones as illustrated in **Figure 3**. While the LSECs within these zones share common features, they do differ from one another. (Mak and Shin 2021) In the periportal zone, the liver sinusoids receive blood from the portal vein and hepatic artery supplying nutrients and oxygen, respectively. The LSECs of periportal zone have fewer fenestrae, but the openings are relatively wider. (Poisson et al. 2017) Endothelial cell progenitors reside in the periportal zone, as do the haematopoietic progenitors during the foetal period (Wakabayashi et al. 2018; Khan et al. 2016). The following zone is called the midlobular zone where the LSECs are specialized to clear the blood by scavenging different types of particles, such as viruses, immune complexes, and lipopolysaccharides. Notably, the LSECs in the midlobular zone can also act as antigen-presenting cells. They are identified by the intensive expression of receptors related to scavenging ability, such as macrophage mannose receptor (CD206), LYVE-1, stabilin-1 and Fcγ receptor IIb (CD32b). (Koch et al. 2021) The third zone is called the pericentral zone, which refers to the area around the central vein that ultimately drains to the inferior vena cava. (Mak and Shin 2021) The LSECs of this region have a higher number of fenestrae, but they are smaller in size compared to the periportal LSEC fenestrae. One of the most important roles of the pericentral LSEC is the maintenance of hepatocyte regeneration through the secretion of the Wingless (Wnt) ligands, Wnt2 and Wnt9b, and the Wnt-signalling enhancer Rspo3. (Koch et al. 2021)

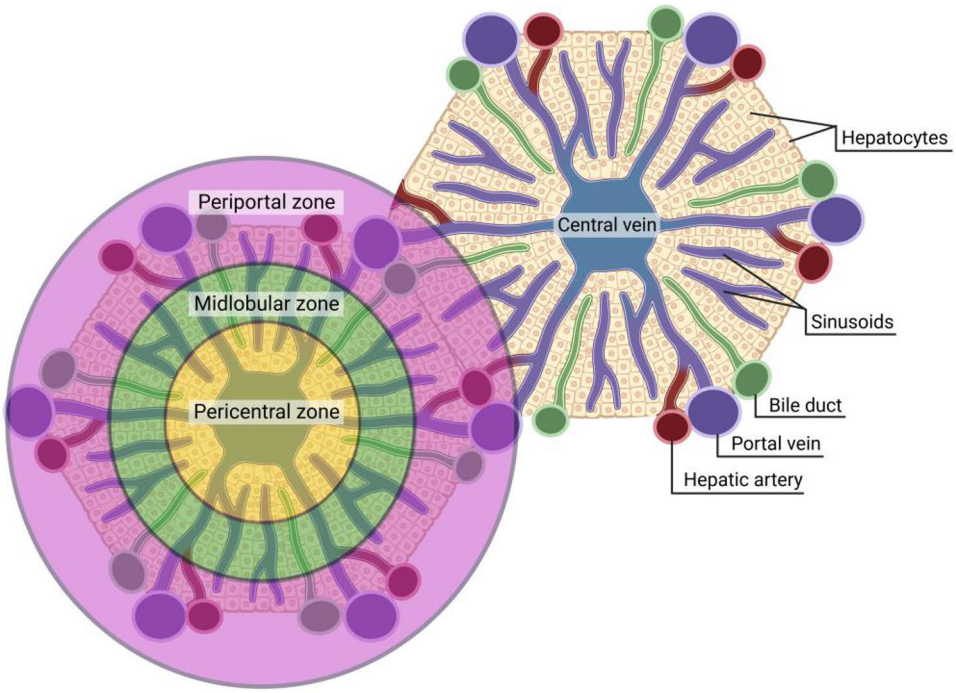

Figure 3. A schematic representation of a liver lobule and the different zones of it. Liver lobule is a hexagon shaped functional and anatomical unit constituted by the hepatocytes, the main parenchymal cell type in the liver. The lobule has a central vein in the middle and a portal triad consisting of a portal vein, a hepatic artery and a bile duct in each corner of the hexagon. Periportal zone (in purple) covers the area around the portal triads that consist of. Pericentral zone (in yellow) is the region surrounding central vein. Midlobular zone (in green) is the sinusoidal area between the periportal and pericentral zones. Created with BioRender.com.

2.1.3.3 Plasmalemma Vesicle Associated Protein (PLVAP)

Opening of the endothelial fenestra, caveola, or TEC can be capped by a diaphragm, which is a sieve structure resembling a cartwheel, as shown by electron micrographs (Bearer and Orci 1985) and as illustrated in **Figure 2**. It is 60-80 nm in diameter and acts as a physical barrier for macromolecules bigger than ca. 60 kDa, permitting passage of hormones and other smaller proteins while prohibiting the crossing of larger molecules like serum albumin (Stan et al. 2012; Chang et al. 2023). The diaphragms are constituted by plasmalemma vesicle-associated protein (PLVAP, also known as PV-1) (Stan et al. 2012). It is an endothelial cell-specific type II transmembrane glycoprotein recognized in mice and humans with MECA-32 and PAL-E antibodies, respectively (Hallmann et al. 1995; Stan 2007; Schlingemann et al. 1985; Niemelä et al. 2005; Keuschnigg et al. 2012). In a diaphragm, extracellular

domains of PLVAP monomers form homodimeric fibrils that protrude from the rim of plasma membrane openings and are joined at the hydrophobic central knob with seven to nine other fibrils (Chang et al. 2023). PLVAP was initially considered a specific marker of blood endothelium, but later the expression was also discovered in the lymphatic endothelial cells in the lymphatic sinus of lymph node (Schlingemann et al. 1985; Rantakari et al. 2015). Notably, PLVAP is known to have a role in the transendothelial migration of leukocytes (Keuschnigg et al. 2009; Rantakari et al. 2015).

PLVAP is the only structural protein needed for the constitution of the diaphragms, which is suggested to be the exclusive expression site for PLVAP (Tkachenko et al. 2012; Stan et al. 2012; Stan, Kubitza, and Palade 1999). Despite decades of research, little is known about the signalling cues behind the up- or down-regulation of PLVAP. In vitro studies have shown that phorbol esters can induce PLVAP (Stan, Tkachenko, and Niesman 2004). It has been demonstrated in the human umbilical vein endothelial cells (HUVEC) that VEGF-A upregulates PLVAP expression; nevertheless, in an immortalised mouse endothelial cell line a similar effect cannot be seen (Strickland et al. 2005; Hnasko et al. 2006). It has been discovered later that VEGF-A signalling via the VEGFR-2 indeed upregulates PLVAP expression in cultured cells, but is not necessary upon phorbol ester-induction (Hamilton, Tse, and Stan 2019). Induction of the Wnt/β-catenin pathway has been reported to downregulate PLVAP (Zhou et al. 2014; Benz et al. 2019; Gastfriend et al. 2021).

In mice, PLVAP is first observed at an mRNA level around E7.0 (Stan, Arden, and Palade 2001). In accordance with the endothelium class, PLVAP is expressed in fenestrated endothelium, while it is absent from continuous endothelium in a steady state (Stan, Kubitza, and Palade 1999). However, acute brain ischemia, for example, has been shown to lead to the breakage of the blood-brain barrier with the emergence of PLVAP expression (Carson-Walter et al. 2005; Shue et al. 2008; Gastfriend et al. 2021). PLVAP constituted diaphragms are known to furnish the fenestrae in the sinusoids of the foetal liver, but in adult mice the fenestrae of the liver sinusoidal endothelium are open (Braet and Wisse 2002). Notably, PLVAP expression has also been linked to some pathologies. It is upregulated in the gut blood endothelium upon *Salmonella* infection and in the vasculature of most tumour types, for instance (Spadoni et al. 2015; Zeng et al. 2023).

PLVAP deficiency in mice leads to increased permeability of blood vasculature at the sites where the diaphragms are normally found, such as the intestines and kidneys (Stan et al. 2012). *Plvap*$^{-/-}$ mice suffer from hypoproteinaemia, oedema in tissues and ascites, and accumulation of triglycerides in the plasma (Stan et al. 2012; Herrnberger et al. 2014). They are also growth retarded and die at the age of one to four months when the *Plvap* deletion is performed on mixed genetic background.

Notably, PLVAP deficiency causes total or high embryo lethality in pure inbred C57BL/6 or mixed backgrounds, respectively, suggesting that PLVAP plays an integral part in ontogeny. (Herrnberger, Seitz, et al. 2012; Rantakari et al. 2015; Stan et al. 2012) Few case studies have shown that patients with mutations in *Plvap* suffer from fatal protein-losing enteropathy, underlining the importance of intact PLVAP expression in humans (Gorukmez, Gorukmez, and Demiroren 2019; Broekaert et al. 2018; Kurolap et al. 2018; Elkadri et al. 2015).

2.1.4 Tissue-resident macrophages

Macrophages, as named by Ilya Metchnikov in the late 1800s, are generally known as tissue-scavenging immune cells taking action against pathogens intruding the body. Now, however, macrophages are known to be involved not only in immunological phenomena but also in the tissue morphogenesis and maintenance of tissue homeostasis both in health and disease. These cells, named tissue-resident macrophages, can generally be described as long-lived, self-renewed, and having extended residency in their target tissue. Even though the majority of the macrophages in adult tissues share common origins, they display high heterogeneity. Even within one organ, macrophages can localize to different compartments and conduct distinct functions specific to their niche. This is due to the high plasticity of the cell type and its sensitivity to microenvironmental cues, such as chemotactic signals or cytokines received through intercellular communication or by delivery to the tissue via blood. Macrophages receive signals via the engulfment of apoptotic, senescent, and dead cells, and phagocytosis of material that can modulate their phenotype from proinflammatory to immunosuppressive. (Blériot, Chakarov, and Ginhoux 2020; Guilliams et al. 2020) They can be identified by their expression for certain surface antigens. F4/80 (Adhesion G Protein-Coupled Receptor E1) encoded by *Adgre1* is one of the best-known antigens expressed on most of the murine resident macrophages, and it has been widely used to identify macrophages ever since its discovery (Austyn and Gordon 1981; Morris, Graham, and Gordon 1991; Schulz et al. 2012). In humans, F4/80 is expressed in eosinophils, but is undetectable in macrophages. CD68, which is also expressed by murine macrophages, has been considered as a pan marker of human macrophages. (Wei et al. 2023; Ráki et al. 2006) To further immunophenotype subpopulations of macrophages, a growing number of surface markers have been discovered, and the complexity expands as the different single-cell techniques are developed and utilized (Blériot, Chakarov, and Ginhoux 2020).

2.1.4.1 Seeding of resident macrophages

The seeding of the tissues by resident macrophages are thought to occur at least in three distinct waves of haematopoiesis. As described earlier in this thesis, the first waves of haematopoiesis emerge during the foetal era. The primitive wave in the extra embryonic yolk sac gives rise to the first macrophages. The second wave, also taking place in the yolk sac, constitutes the EMPs and LMPs that expand and differentiate in the foetal liver. Finally, the third wave refers to postnatal haematopoiesis by the HSCs residing in the bone marrow.

Historically, resident macrophages in adults are thought to originate mainly from the bone marrow-derived monocytes (van Furth et al. 1972). Hints that there is more to the story were provided when Merad et al. (2002) showed that resident macrophages in the dermis, named Langerhans cells, were maintained through local self-renewal rather than replenishment from circulating progenitors in homeostasis. Thereafter, the dogma was shattered when Ginhoux et al. (2010) found that adult microglia are predominantly of embryonic origin. Their postirradiation bone marrow transplantation experiment suggests that circulating monocytes nominally contribute to the microglia population in homeostasis. Concurrent results were obtained from the extensive parabiosis experiments, where circulatory systems of two mice having different allotypes of the panleukocyte protein, CD45, were connected in vivo (Ginhoux et al. 2010). Moreover, lineage-tracing studies have shown that microglia are specifically constituted by primitive macrophage progenitors from the yolk sac (Mildner et al. 2007; Ajami et al. 2007; Ginhoux et al. 2010). After these discoveries, the research on the origins of tissue macrophages has been growing rapidly.

According to lineage-tracing studies in mice, primitive macrophages seem to populate all the tissues during early foetal development, but a remarkable dilution of these tracked cells is observed in the majority of the tissues starting after E12.5 (Gomez Perdiguero et al. 2014; Hoeffel et al. 2015). Around that time, the foetal liver, which has just been colonized by the EMPs, LMPs, and HSCs, begins to produce differentiated blood cells, including monocyte precursors, into circulation (Hoeffel and Ginhoux 2018). The specific progenitor cell for those monocytes has been a topic for lively discussion. EMPs have been proposed to constitute a vast majority of them, while a small but distinct contribution comes from the LMPs as well (Hoeffel et al. 2015; Böiers et al. 2013). The role of HSCs in the constitution of leukocyte and thereby foetal macrophage populations seems negligible (Hoeffel and Ginhoux 2018). These theories have been supported by the latest single-cell RNA sequencing data (Ceccacci et al. 2023). Foetal monocytes, identified by the distinct expression of Ly6C and CCR2, are observed in several peripheral tissues, including the lung, kidney, and skin by E14.5. Later, they differentiate into macrophages, detected as an upregulation of F4/80 and downregulation of Ly6C expression. Notably, the brain is not infiltrated by the foetal monocyte-derived macrophages, but

elsewhere they outnumber the yolk sac-derived macrophages by E16.5. (Hoeffel et al. 2015) These foetal liver-originated macrophages persist and constitute the resident macrophage populations in various tissues, such as lung alveolar macrophages (Guilliams et al. 2013), Kupffer cells in the liver (Yona et al. 2013), and part of the cardiac macrophages (Epelman et al. 2014).

Although many tissue-resident macrophage populations originate during the foetal era, they can be accompanied or, in some cases, replenished by new macrophage populations in the postnatal life. For example, the resident macrophage pool in the colon is largely and constantly replenished by the circulating monocytes (Bain et al. 2014). It has been shown that in selected tissues, such as the lung, spleen, kidney, and peritoneum, monocytes start to replenish the macrophage pool only later in life, and, moreover, do so in a sex-biased manner (in peritoneum) (Liu et al. 2019; Bain et al. 2016). It is well established, at least in the liver and lungs, that an emptied tissue niche can be replenished by the bone marrow-derived monocytes. They are able to acquire the phenotype and functionalities of the depleted cells, although this sort of re-education is completed only after months of the seeding. (van de Laar et al. 2016; Scott et al. 2016) Notably, the latest published data indicate that the layered program of haematopoiesis reviewed here is highly conserved and is identifiable in humans as well (Bian et al. 2020). The main haematopoietic sources of the resident macrophages are illustrated in **Figure 4**.

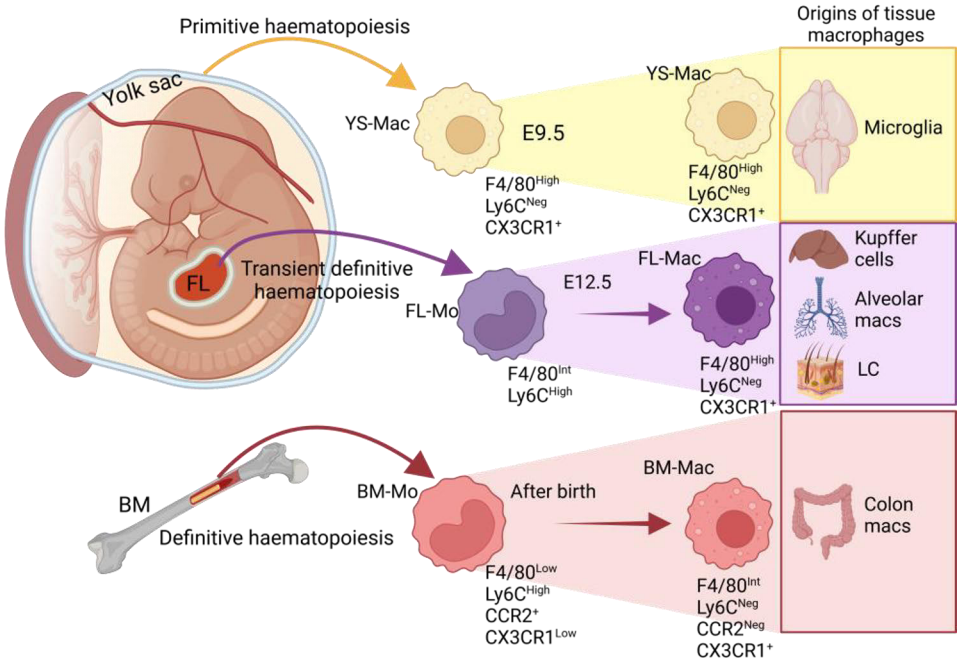

Figure 4. A schematic representation of the haematopoietic waves producing resident macrophages. The first macrophages emerge around E9.5 during the primitive haematopoiesis in the yolk sac and they are the first macrophages to seed the tissues. Microglia in the brain are constituted mainly by these yolk sac-derived macrophages. Transient definitive haematopoiesis initiates in the foetal liver (FL) and produces foetal monocytes into circulation around E12.5. The monocytic precursors seed the tissues and only then mature into macrophages. Resident macrophages in the liver, lungs and skin, for example, are derived from the foetal liver. Finally, after birth the definitive haematopoiesis in the bone marrow (BM) starts to produce circulating monocytes that can seed the tissues and mature into resident macrophages as well. Circulating monocyte-derived macrophages are found, for example, in the colon where they are under constant and rapid turnover. The expression of key markers in the yolk sac-, foetal liver- and bone marrow-derived macrophages (YS-Mac, FL-Mac and BM-Mac, respectively) and their monocyte precursors (foetal liver-derived monocytes, FL-MO, bone marrow-derived monocytes, BM-MO) are also shown. Langerhans cells, LC. Created with BioRender.com.

2.1.4.2 Tissue imprinting of macrophages

Resident macrophages are imprinted by their tissue niche, which means that the other cell types in the tissue are educating the macrophages to conduct tasks specifically needed in the niche. The stromal cells, including epithelial and endothelial cells, pericytes, fibroblasts, and other leukocytes, produce growth factors and cytokines

required for the recruitment, survival, and differentiation of resident macrophages. Colony-stimulating factors 1 and 2 (CSF1 and CSF2, respectively) and interleukin 34 (IL-34) are among the best known trophic factors related to resident macrophage biology. Of these, CSF1 is needed for the maintenance of most resident macrophage populations, and those populations show partial selectivity for the membrane-bound or cell-secreted soluble isoforms of the growth factor. (Guilliams et al. 2020) For example, the Kupffer cells seem dependent on circulating CSF1 (Cecchini et al. 1994), while macrophages in the bladder suffice on local CSF1 supply (Dai et al. 2004). The membrane-bound growth factor has been suggested to determine the exact niche for some resident macrophages. Hence, they would make close contact with the growth factor supplying cells and consequently inhibit other macrophages from occupying the space deprived of the essential nutrient. (Guilliams et al. 2020) These kind of cell-cell pairs of macrophages and fibroblasts have been proven to form in vitro (Zhou et al. 2018).

As the niche has been made attractive for the macrophages to settle in, they are then conditioned to serve for the benefit of the tissue which refers to functions beyond immune surveillance and phagocytosing excess material. The conditioning is governed via signals that induce the expression of specific transcription factors that guide the macrophages to function in the desired manner. The macrophage imprinting in the tissue can be observed on a transcriptional level as certain transcription factors enhance the expression of certain genes. (Gosselin et al. 2014; Lavin et al. 2014) For instance, foetal monocytes in the prenatal lung require induction of peroxisome proliferator-activated receptor-γ (PPARγ) by CSF2 to mature into alveolar macrophages (Schneider et al. 2014). Besides cell-cell signalling, macrophages adapt tissue-specific functions through phagocytosis. An example of this is the iron recycling macrophages in the spleen. They take up senescent erythrocytes containing haem, which leads to degradation of the transcriptional repressor controlling SPI-C transcription factor, which, in turn, promotes macrophage specialization to iron recycling. (Haldar et al. 2014) Other examples of tissue-specific functions of macrophages are the regulation of electrical activity of cardiomyocytes in the heart (Hulsmans et al. 2017) and surfactant clearance by alveolar macrophages in the lungs (Suzuki et al. 2008).

2.1.5 Tools for tracing and depleting macrophages

2.1.5.1 Lineage tracing mouse models

Lineage tracing is a powerful tool for studying the ontogeny of macrophages. The Cre recombinase system is one of the most utilized strategies in the lineage tracing. To put it simply, the *Cre*-cassette is inserted into the genome to be expressed under

the control of a specific gene promoter. The expressed Cre then recognizes the specific nucleotide sequence called locus of x-over in P1 bacteriophage (*loxP*) and induces recombination between two *loxP* sites. If the two *loxP* sites are directly repeated the DNA fragment flanked by them is deleted. (Kim et al. 2018; Metzger et al. 1995) For example, when the two *loxP* sites are flanking a stop codon inserted between a reporter gene, such as enhanced yellow fluorescent protein (EYFP) coding gene, and its promoter, the Cre deletes the stop codon allowing transcription of the reporter gene. All the cells expressing the gene with *Cre*-cassette insertion and their progeny will stably express the reporter. (Srinivas et al. 2001) The Cre recombinase can be further controlled by fusing it with the oestrogen receptor (ER). The Cre-ER fusion protein localizes to the cytosol and can only translocate to the nucleus for recombinase activity in the presence of Tamoxifen, a selective ER modulator. This approach enables the timed labelling of the cells and their progeny. (Kim et al. 2018) A schematic representation of a Tamoxifen inducible Cre reporter system is illustrated in **Figure 5**.

Lineage tracing mouse models have been used to trace various leukocyte lineages. For example, early haematopoietic progenitors can be targeted for lineage tracing by expressing Cre under the control of genes expressed in the haemogenic endothelium. To label specific progenitor cells derived from the haemogenic endothelium, the Tamoxifen inducible *Cre-ER*-reporter mice have been utilized. One of the mouse strains used in this manner is *Runx1*-tied *Cre-ER*. *Runx1* is expressed in the endothelial cell upon transitioning to a haematopoietic cell. Therefore, yolk sac-derived primitive macrophages are labelled when Cre expression is induced at E7.0-7.5, whereas EMPs and premature HSCs emerging from the haemogenic endothelium of the yolk sac and para-aortic splanchnopleura, respectively, are tagged if the Cre is induced after E8.0. (Chen et al. 2009; Samokhvalov, Samokhvalova, and Nishikawa 2007; Ginhoux et al. 2010) Another Cre driver used in a similar manner is *Tie2* which is expressed in endothelial cells as well as in HSCs (Busch et al. 2015; Gomez Perdiguero et al. 2014).

Whereas previously mentioned lineage tracing models target the endothelial precursor of haematopoietic cells, other models target the haematopoietic cells directly. For instance, *Flt3* (Flk2) -*Cre* mice can be used to label the multipotent haematopoietic progenitors without temporal induction (Benz et al. 2008; Christensen and Weissman 2001; Hoeffel et al. 2015). *Kit*, which is expressed by the haematopoietic stem and progenitor cells, is utilized again in a timed manner to target primitive and definitive progenitors distinctively (Sheng, Ruedl, and Karjalainen 2015; McGrath, Frame, and Palis 2015). Some lineage tracing models target even further lineage committed cells. CSF1 and its receptor CSF1R (CD115) are known for their role in monocyte and macrophage survival and differentiation (Guilliams et al. 2020). Notably, the *Csf1r* expression was also discovered in early

macrophage progenitors (Ginhoux et al. 2010) and EMPs (Gomez Perdiguero et al. 2014) emerging from the yolk sac. Subsequently, Tamoxifen inducible *Csf1r-Mer-iCre-Mer* mice have been used to track yolk sac-derived macrophages, which can be labelled by inducing recombination at E8.5 (Ginhoux et al. 2010; Hoeffel et al. 2015; Gomez Perdiguero et al. 2014). CX3CR1, expressed by monocytes and macrophages, has also been proven useful in tracing yolk sac-derived macrophages and studying the fate of monocytes. For those purposes, there are both inducible and spontaneous Cre mice available on top of the original reporter mouse constitutively expressing GFP instead of CX3CR1. (Jung et al. 2000; Yona et al. 2013; Goldmann et al. 2016)

The membrane-spanning 4-domains, subfamily A, member 3 (Ms4a3) was identified to be differentially expressed in granulocyte-monocyte-progenitor (GMP) and monocyte-dendritic cell progenitor, which are precursors for monocytes. Ms4a3-Cre and its inducible counterpart *Ms4a3*-CreERT2 mouse models have been utilized to trace adult bone marrow GMP-derived monocytes. (Liu et al. 2019) Another fate-mapping model relies on CCR2, which is expressed by monocytes and macrophages and has been functionally related to monocyte egress from bone marrow (Serbina and Pamer 2006). Although foetal monocytes express CCR2 as well, their migration does not depend on it (Hoeffel et al. 2015). Hence the inducible *Ccr2*-CreERT has been exploited in the fate mapping of foetal liver- and adult bone marrow-derived monocytes and their progenies (Hoeffel et al. 2015; Croxford et al. 2015; Dick et al. 2022). Despite the undeniable advantages of the lineage tracing models, they do have some limitations as well. Those include unexpected expression of the target genes causing labelling in off-target lineages. In inducible models, the Tamoxifen dosage must be balanced to maintain pregnancy and ensure adequate induction, which rarely if ever achieves complete labelling efficiency. Also the bioavailability of Tamoxifen may vary within one litter and between litters. (McGrath, Frame, and Palis 2015; Danielian et al. 1998)

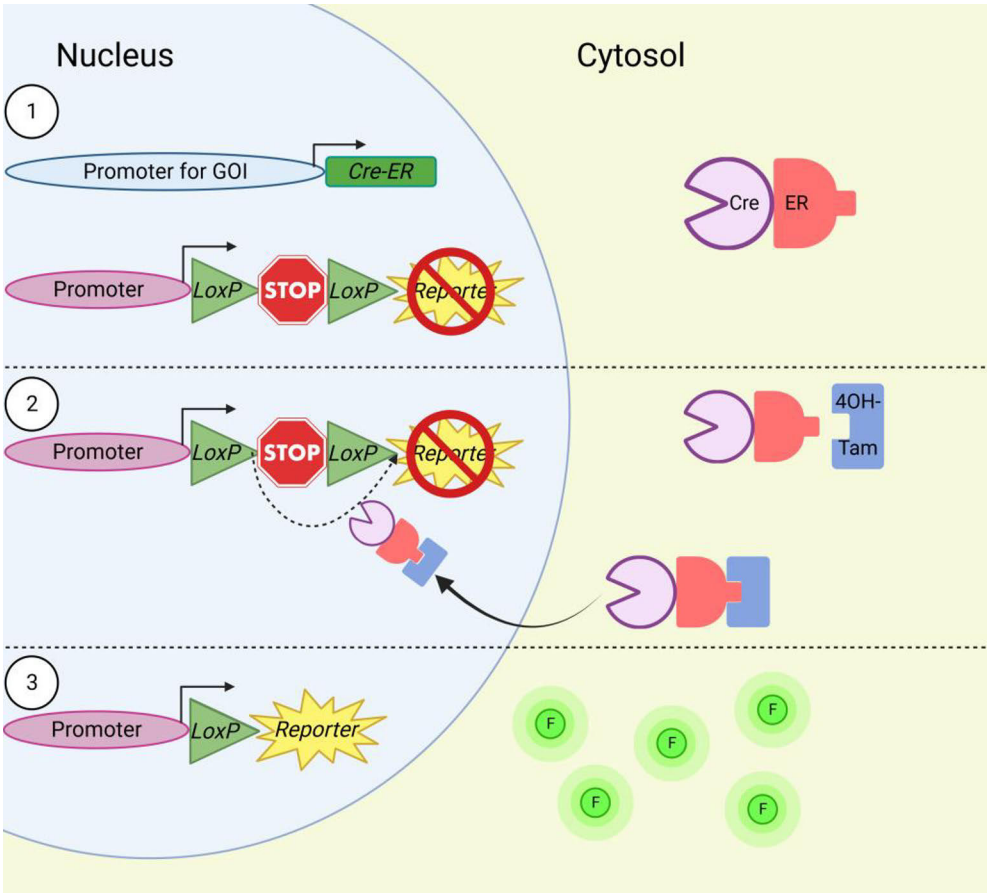

Figure 5. A schematic representation of a Tamoxifen inducible Cre reporter system. (1) First the Cre-oestrogen receptor (ER) fusion protein is expressed under the control of a gene of interest (GOI) and its promoter and located to cytosol. The expression of the reporter gene is blocked by the stop codon. (2) Then Tamoxifen and its metabolite 4-hydroxytamoxifen (4OH-Tam) is introduced to the system. 4OH-Tam binds to the ER and induces the translocation of the fusion protein to the nucleus, where Cre recognizes the directly repeated *loxP* sites and deletes the stop codon flanked by them. (3) Consequently, the reporter gene encoding a fluorophore (F) is expressed. Created with BioRender.com.

2.1.5.2 Macrophage deficient mouse models

Tissue-specific functions of macrophages are relatively difficult to test in vivo. Therefore, hints of their actions must be acquired by different methods. One method is to use genetically modified mice, where genes critical for monocyte and macrophage survival, differentiation, migration, or function are either partially or entirely deleted or interfered with through point mutations. One example of the mutated genes leading to macrophage deficiency is the osteopetrotic (*CSF1*^{op}) mouse strain, which lacks a biologically active CSF1 (Wiktor-Jedrzejczak et al. 1990). These mice suffer from a systemic lack of macrophages, but the most apparent macrophage population affected is the osteoclasts, as the skeletal structures appear fragile and deformed (Naito et al. 1991). Nevertheless, some macrophages, such as microglia, are observed in only partially reduced numbers (Blevins and Fedoroff 1995). Notably, the same defects observed in *CSF1*^{op} mice, with increased severity, are seen in CSF1-receptor deficient mice (Dai et al. 2002).

Two widely used models to assess monocyte functions in tissues are *Ccr2*$^{-/-}$ and *Nur77*$^{-/-}$ mice. CCR2 is known to play an essential role in inflammation and is related to Ly6C^{high} monocyte emigration from the bone marrow. This egress is critically impaired in *Ccr2*$^{-/-}$ mice leading to decreased numbers of circulating Ly6C^{high} monocytes. (Boring et al. 1997; Serbina and Pamer 2006) Another mouse model with altered monocyte numbers is *Nur77*$^{-/-}$ (also known as the nuclear receptor subfamily 4 group A member 1, abbreviated *Nr4a1*). These mice have significantly fewer Ly6C$^{low/-}$ patrolling monocytes in the blood as they go to apoptosis in the bone marrow. (Hanna et al. 2011) Notably, *Nur77*$^{-/-}$ mice were utilized in the discovery of a thymic resident macrophage population (Tacke et al. 2015).

Deletion of specific cells can also be achieved by utilizing the Cre-lox system mentioned in the previous chapter. Instead of a reporter, gene a gene encoding diphtheria toxin subunit A (DTA) can be inserted downstream of the *loxP* flanked stop codon. The DTA, once in the cytosol, impairs protein synthesis and causes apoptosis. Hence, the Cre expressing cells are ablated. (Ivanova et al. 2005) Alternatively, instead of DTA, a diphtheria toxin receptor (DTR) can be expressed through Cre-lox recombination. Mice do not express DTR naturally and are therefore resistant to diphtheria toxin. Hence diphtheria toxin administration leads to specific ablation of cells with the Cre activated DTR. (Buch et al. 2005)

Macrophage deficient mouse models are often used to study the in vivo functions of the macrophages, but caution must be taken when interpreting the data for few reasons. First, the depletion/interference might not be complete due to alternative mechanisms switching on to maintain survival/function. Second, macrophage deficiency achieved with the genetic models is usually systemic, thus causalities

between the observed physiological or anatomical effects and the lack of specific macrophage population requires supporting studies.

2.1.5.3 Other strategies to deplete macrophages

Clodronate liposomes are one of the most commonly used particles to deplete macrophages. Liposomes are used as a vehicle to deliver clodronate or other depleting molecules to macrophages, which ingest such particles. When ingested and digested in the macrophages, the clodronate molecules are released, which induces apoptosis in the target cell. As different barriers and functions can prevent even distribution of the liposomes, the route of administration needs to be carefully considered. While intravenous injection efficiently depletes the Kupffer cells in the liver and red pulp macrophages in the spleen, intratracheal or intranasal administration is required for effective ablation of alveolar macrophages in the lungs. (van Rooijen and Hendrikx 2010) Of note, the blood monocytes are abolished as well by intravenous administration of clodronate liposomes (Sunderkötter et al. 2004).

Neutralizing or blocking antibodies are also available to cause macrophage ablation. Anti-CSF1R blocking antibody has been used to deplete yolk sac-derived macrophages when given to a pregnant dam at E6.5. Notably, this approach has no apparent impact on foetal liver-derived macrophages. (Squarzoni et al. 2014) When adult mice are treated with CSF1R antibody it depletes resident macrophage subpopulations selectively in specific tissues (MacDonald et al. 2010). Resident macrophages of Langerhans islets in the pancreas and capsular macrophages in the liver are abolished (Carrero et al. 2017; Sierro et al. 2017). Nevertheless, Ly6C^{high} circulating monocyte numbers are decreased only little or are unaffected (MacDonald et al. 2010). Neutralizing antibodies against the two CSF1R ligands, CSF1 and IL-34, can be used to specifically deplete microglia in white and grey matter, respectively (Easley-Neal et al. 2019). Additionally, the CSF1 antibody was shown to deplete the Ly6C$^{low/–}$ monocytes (Louis et al. 2015). These are just examples of antibodies used to deplete monocytes and macrophages; while many others do exist, they are not mentioned here. Notably, as with macrophage deficient mouse models, antibody or chemical treatments lead to a systemic depletion of the macrophages or other cells, therefore any direct conclusions on the specific macrophage subpopulations should not be drawn based purely on them.

2.2 Testis

The testes are the male reproductive glands that perform steroidogenesis as an endocrine and gametogenesis as an exocrine function. Thus, the main responsibility of the testes is to produce steroids and to produce and store sperm for reproduction. A short introduction to the organ, its development, and the immunological status is given in the following chapters.

2.2.1 Structure and function of the testis

The testis is surrounded and isolated from the rest of the body by a capsule which consists of three membranous layers including the outermost tunica vaginalis, a fibrous membrane called the tunica albuginea and the vascular layer called tunica vasculosa. Inside the capsule, the testis can be divided into two functionally different compartments, the interstitium and the seminiferous tubules, that are also illustrated in **Figure 6**. The seminiferous tubules contain the Sertoli cells, which constitute the seminiferous epithelium, and the developing sperm cells. The interstitial space between the coiling tubules accommodates blood and lymphatic vessels and the steroidogenic Leydig cells, for example. The different cell types of testis have distinct localization and specific functions that support the integrity of spermatogenesis. (Creasy and Chapin 2013)

The spermatogonial stem cells (SSC) are lodged in the basement membrane of the seminiferous epithelium at specific sites called stem cell niches, which have been shown to locate in the close proximity of the testicular blood vessels and interstitial tissue (Yoshida, Sukeno, and Nabeshima 2007). In there, the few true stem cells are mitotically proliferating for self-renewal but also for the production of generations of spermatogonial progenitors. The long-lived SSCs are identified as single cells while the SSC progenies are interconnected via cytoplasmic bridges forming syncytia of two to 16 cells. (Mäkelä and Hobbs 2019) Notably, the syncytia may be fragmented and the resulting single cells can revert to the long-lived ancestor state when needed (Hara et al. 2014). The SSC self-renewal is regulated by many factors including glial cell line-derived neurotrophic factor (GDNF) produced by Sertoli cells and testicular endothelial cells (Meng et al. 2000; Bhang et al. 2018). The differentiation, in turn, is induced by retinoic acid signalling (Mäkelä and Hobbs 2019; Van Pelt and De Rooij 1990). Spermatogenesis proceeds and the differentiated spermatogonia mature into spermatocytes which duplicate their DNA content and enter the meiotic cell cycle. The second meiotic division produces four haploid cells called rounded spermatids. Those cells undergo morphological changes where new structures such as acrosome and flagella are acquired, and the histones are substituted by protamines. The spermatozoa then migrate to the epididymis to complete the

maturation. (Nishimura and L'Hernault 2017; Mäkelä and Hobbs 2019; Creasy and Chapin 2013)

The SSCs and all of their progeny are nursed by Sertoli cells. Sertoli cells are identifiable by their nucleus containing large nucleolus and located near the basal lamina of seminiferous tubules and the peripheral arm like branches that are wrapping around the sperm cells. (França et al. 2016) The seminiferous epithelium is divided into two compartments by the tight junctions between Sertoli cells. This structure is called the blood-testis-barrier (BTB) or Sertoli cell barrier, which prohibits the passage of molecules and leukocytes, for example. The junctions are mainly constituted by occludin, claudins, and junctional adhesion molecules. The basal compartment separated by the BTB contains the SSCs and their premeiotic progeny while the adluminal compartment accommodates the spermatids. (Mruk and Cheng 2015) While BTB is indispensable for intact spermatogenesis, it is unnecessary for the prevention of auto-immunogenic response. Sertoli cells notably provide spermatogenesis not only with structural support but also with paracrine secretion of factors needed for the maintenance and differentiation of germ cells, such as GDNF and retinoic acid, respectively. (França et al. 2016; Meng et al. 2000)

The cells on the borderline of seminiferous tubules are peritubular myoid cells (PMC). In rodents, PMCs form a monolayer, but in humans, there are multiple layers. They are smooth muscle cell -like and perform peristaltic contractions which enable sperm movement towards a common collection site for sperm cells called rete-testis. (Creasy and Chapin 2013) PMCs constitute another semipermeable barrier for the contents of the seminiferous tubules allowing passage of molecules but preventing cell infiltration (Mruk and Cheng 2015). Besides structural support and barrier function, PMCs also produce GDNF and thereby contribute to the SSC niche (Chen et al. 2014).

Leydig cells reside in the interstitium of the testis, adjacent to the vasculature and the PMCs (Creasy and Chapin 2013). Leydig cells are considered long-lived with no major turnover. They are the steroidogenic responders for the hypothalamic–pituitary–gonad axis at the testicular end. They express receptors for luteinizing hormone (LH) and consequently synthesize and secrete testosterone, which is an absolute necessity for intact spermatogenesis. (Zirkin and Papadopoulos 2018) Leydig cells also secrete vasopressin and oxytocin to induce peristaltic contractions by the PMCs (Heinrich and DeFalco 2020). Notably, Leydig cells are also major producers of CSF1, which has been shown in vitro to promote the stemness of the SSCs expressing CSF1R. However, the effect of CSF1 in SSC biology in vivo remains to be elucidated. (Oatley et al. 2009; Kokkinaki et al. 2009)

Other important interstitial cell types in the testis include mesenchymal progenitors that can give rise to Leydig cells (Chen et al. 2010). The blood vessel endothelial cells in the testis, called testicular endothelial cells, have been shown to

contribute to SSC niche by expressing GDNF, among other factors, and are able to rescue spermatogenesis after the SSC depletion by chemotherapy in a mouse model (Bhang et al. 2018).

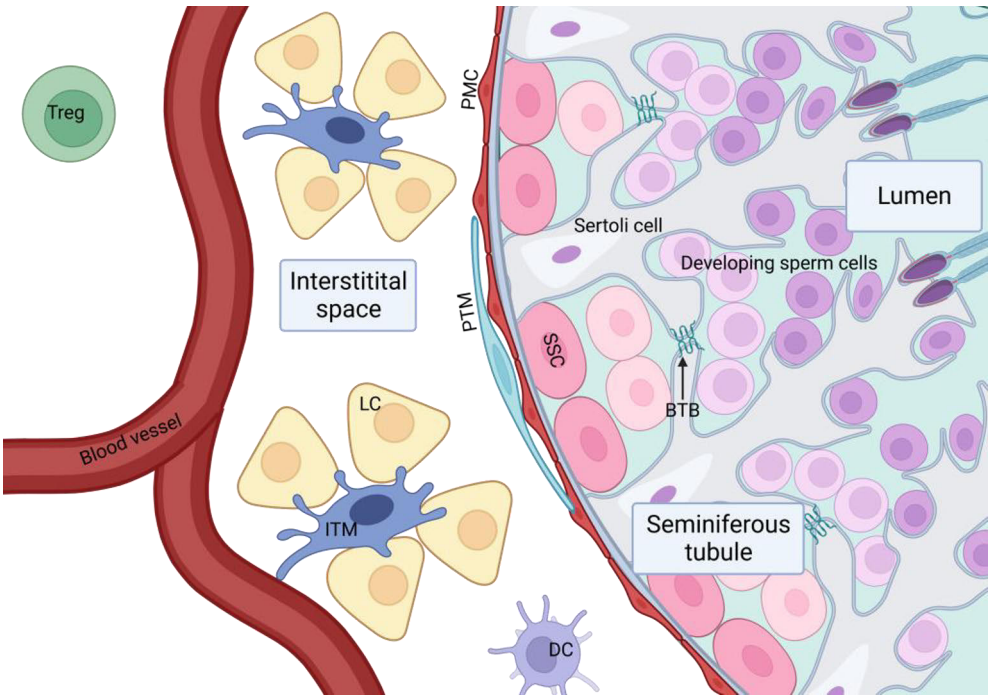

Figure 6. A schematic view of the testis interstitial space, seminiferous tubules and their cell types. Interstitial space consists mainly of the Leydig cells (LC) intermingling with the interstitial macrophages (ITM). Other immune cells found in the interstitium are dendritic cells (DC) and regulatory T cells (Treg). Outer layer of seminiferous tubule is lined by the peritubular myoid cells (PMC) and the peritubular macrophages (PTM) lying in their immediate vicinity. Basal membrane produced by Sertoli cells separates PMCs from spermatogonial stem cells whose progenies are then transported further to the lumen of the seminiferous tubules while they mature. Sertoli cells provide physical support and nutrients for the developing sperm cells but also regulate the access of cells and molecular components to different compartments of the seminiferous tubule via multiple tight junctions constituted blood-testis-barrier (BTB). Created with BioRender.com.

2.2.2 Testis development

Coelomic epithelial cells in the ventromedial surface of the mesonephros start to proliferate around E9.5 in mice, developing a gonadal ridge. The bipotential gonads are formed when the coelomic epithelial cells differentiate to constitute the supporting cell precursors and steroidogenic cell precursors. (Xie et al. 2022)

Notably, the very first primordial germ cells emerge in the endoderm of the extra embryonic yolk sac around E6.0-E7.0. They translocate to the developing bipotential gonads between E10.0 and E11.5. The testicular differentiation begins as directed by *sex-determining region Y* (*Sry*) expression by the early somatic cells. This is further enhanced by pre-Sertoli cells and their expression for Sry box 9 (*Sox9*) and anti-Müllerian hormone (AMH), which is responsible for the regression of Müllerian ducts giving rise to the female reproductive tract. Hence, the pre-Sertoli cells are responsible for the differentiation of the testis. The differentiation comprises among other events, the formation of testicular cords that encase both pre-Sertoli cells and germ cells but leave out other interstitial cells such as the foetal Leydig cells. After birth, seminiferous tubules are formed, and germ cells differentiate into SSCs, foetal Leydig cells are replaced by adult Leydig cells, and pre-Sertoli cells enter the cell cycle. (Mäkelä et al. 2019) The pre-Sertoli cells proliferate until gradually halted by the postnatal day (P) 17 (Vergouwen et al. 1991). During this time, they also mature and constitute the BTB. The maturation of Sertoli cells and the emergence of adult Leydig cells enable the onset of spermatogenesis right after birth. (Mäkelä et al. 2019)

2.2.3 Immune landscape of testis

The testis is one of the few sites in the body that is considered immune privileged. The other such sites are the brain and placenta, for instance. Meiotic and postmeiotic sperm cells first appear after the establishment of self-tolerance. Hence, they express previously unknown antigens which are considered non-self by the immune surveillance. Therefore, the testis niche must be highly regulated to protect the gametes from both pathogens and immune cells. BTB and PMCs provide physical protection for meiotic to postmeiotic and premeiotic sperm cells, respectively. The Sertoli cells secrete cytokines such as transforming growth factor beta (TGFβ) and activins along with other molecules that promote immunosuppression. Furthermore, they phagocytose apoptotic sperm cells to prevent the danger signal cascade initiation. (Fijak, Bhushan, and Meinhardt 2016) As shown by accepted tissue allografts transplanted into the interstitium of the testis, the immune privilege comprises the whole organ and not only the seminiferous tubules (Head, Neaves, and Billingham 1983). Notably, the steroid hormones, namely testosterone produced by Leydig cells, also contribute to the immune privilege by inducing Sertoli cells to express immunosuppressive factors (Fijak, Bhushan, and Meinhardt 2016).

Despite the high immune regulation in the testis, multiple leukocyte types are present in the interstitial space. Notably, seminiferous tubules are mainly devoid of any leukocytes in a steady state, excluding the regions close to rete testis, and infiltration is only detected under pathological circumstances. (Shechter, London,

and Schwartz 2013) Unlike the brain, for example, the testis encompasses a network of lymphatic vessels for lymphocytes and dendritic cells to migrate to the testis-draining lymph node. Indeed, T lymphocytes, of which $CD8^+$ cells represent the majority, are present in the interstitial space of the testis in an unchallenged state. Moreover, some regulatory T cells have been observed in the testis, especially in the testis-draining lymph node. Also, dendritic cells with the tolerogenic phenotype and incapacity to induce naïve T cell proliferation or priming reside in the testis in a steady state. Under pathological conditions, whether it be a pathogen attack or an auto-antigenic trigger, both T cells and DCs acquire pro-inflammatory status. (Bhushan et al. 2020) Other immune cell types observed in testis include mast cells, natural killer cells, and eosinophils, whose ratios vary among species (Tompkins et al. 1998; Anton et al. 1998).

Macrophages represent the largest immune cell population in the testis. They can be divided into two subpopulations by their morphology and localization. (DeFalco et al. 2015; Mossadegh-Keller et al. 2017) Interstitial macrophages mingle with the Leydig cells and have been shown to have physical intercellular connections with them in rats (Hutson 1992). The exact nature of the relationship between Leydig cells and macrophages remains to be fully elucidated, but as the Leydig cells are producing CSF1, they likely provide a compelling niche of residence for the macrophages (Fijak, Bhushan, and Meinhardt 2016). On the other hand, the androgens have been shown to downregulate the production of pro-inflammatory cytokines such as TNF and upregulate IL-10, an anti-inflammatory cytokine (Agostino et al. 1999). Indeed, the transcriptome profile of the interstitial macrophages indicated the presence of an immune-suppressive phenotype (Mossadegh-Keller et al. 2017). Notably, the macrophages have been proposed to promote Leydig cell differentiation and development postnatally and to provide 25-hydroxycholesterol for testosterone synthesis by Leydig cells with a paracrine negative feedback loop (Gaytan et al. 1994; Nes et al. 2000; Lukyanenko, Chen, and Hutson 2002). Another macrophage population attached to a seminiferous tubule wall has been acknowledged earlier, but was described in detail only recently (Hume et al. 1984; Itoh et al. 1995; DeFalco et al. 2015). The cells with dendritic cell-like morphology and distinct expression of MHC II were observed laying on seminiferous tubules alongside with PMCs. They have been proposed to have a regulatory role in spermatogenesis as they express retinol dehydrogenase 10 (RDH10), which is an important enzyme for retinoic acid synthesis. (DeFalco et al. 2015)

3 Aims

Endothelial cells lining the blood vasculature represent the important interface between tissue stroma and blood. They are in a key position to regulate the trafficking of macromolecules and cell migration. PLVAP has been used as a pan blood endothelial marker, and its function as a size-selective sieve for macromolecules is appreciated. However, little is known about the other functions it may hold. Specific aims of this thesis were:

1. Investigate the role of PLVAP in foetal monocyte migration.
2. Explore PLVAP expression and functions in the liver sinusoidal endothelial cells throughout development.
3. Elucidate ontogeny of testicular tissue-resident macrophages in a steady state.
4. Examine the significance of testicular tissue-resident macrophages for male fertility.

4 Materials and Methods

4.1 Animal models (I-III)

Mouse models were used in all studies of this thesis. Mice were housed and bred in open or individually ventilated cages under specific pathogen-free conditions at 20-23 °C in a light-dark cycle of 12 hours in the Central Animal Laboratory of the University of Turku. Dry chow and water were available *ad libitum*. All animal experiments were approved by the Ethical Committee for Animal Experimentation in Finland. They were carried out in compliance with the rules and regulations of the Finnish Act on Animal Experimentation (497/2013) respecting the principles of the 3Rs and under the following animal licences: 5587/04.10.07/2014, 6211/04.10.07/2017 and 14685/2020.

All the mouse strains used in this thesis are listed in **Table 1**. Mice were used at indicated ages. Genetically modified mice had wild type and/or littermate controls matched by age, sex, and strain. Both sexes were used for studies I and II, while only males were used for study III. To study prenatal time points, timed mating pairs were established. Females were checked for the vaginal plug in the mornings and the day of detection was determined as embryonic day 0.5 (E0.5).

To activate Cre recombinase in mouse strains expressing Cre-oestrogen receptor fusion protein, the pregnant dams were administered intraperitoneally (i.p.) with 1.5 mg of tamoxifen amended with 0.75 mg progesterone (Sigma–Aldrich) at indicated time points.

Table 1. Mouse strains used in the studies.

MOUSE STRAIN	DESCRIPTION	SUPPLIER/REFERENCE	STUDY
Wild type strains			
C57BL/6NRj	Inbred wild type	Charles River	I-III
C57BL/6JRj	Inbred wild type	Janvier labs or Jackson Laboratory	II-III
BALB/c	Inbred wild type	Janvier Labs	I-II
C57BL/6;129	B6129SF2/J F2 hybrid	Jackson Laboratory	I

MOUSE STRAIN	DESCRIPTION	SUPPLIER/REFERENCE	STUDY
		Stock 101045	
Immunodeficient strains			
Plvap$^{-/-}$	*Plvap*^{tm1Salm} Targeted constitutive *plvap* knockout	(Rantakari et al. 2015)	I-III
Nt5e$^{-/-}$	*Nt5e*^{tm1Lft} Altered leukocyte trafficking	(Thompson et al. 2004)	I
Aoc3$^{-/-}$	*Aoc3*^{tm1Salm} Altered leukocyte trafficking	(Stolen et al. 2005)	I
Cav1$^{-/-}$	*Cav1*^{tm1Mls}/J Lack of caveolae	Jackson Laboratory Stock 004585 (Razani et al. 2001)	I-II
Ccr2$^{-/-}$	B6.129s4-*Ccr2*^{tm1Ifc}/J Deficient monocyte recruitment in inflammation	Jackson Laboratory Stock 004999	III
Nur77$^{-/-}$	B6;129S2-*Nr4a1*^{tm1Jmi}/J Decreased numbers of patrolling monocytes in blood	Jackson Laboratory Stock 006187	III
Csf1^{op}	B6;C3Fe *a/a-Csf1*^{op}/J General macrophage deficiency, monocytopenia and defective bone remodeling	Jackson Laboratory Stock 000231	III
Plvap$^{F/F}$; *Lyve1*$^{Cre+}$	B6;129P2-*Lyve1*$^{tm1.1(EGFP/cre)Cys}$/J Conditional deletion of *Plvap* in *Lyve-1*$^{+}$ cells	(Rantakari et al. 2015) Jackson Laboratory Stock 012601	I
Inducible strains			
PlvapF/F; CAGGCre-ER^{TM}	*Plvap*$^{F/F}$ conditional crossed with B6.Cg-Tg(CAG-cre/Esr1*)5Amc/J	(Rantakari et al. 2015) Jackson Laboratory Stock 004682	I
CX3CR1^{CreERT2}; *R26R-EYFP*	B6.129P2(C)-*Cx3cr1*$^{tm2.1(cre/ERT2)Jung}$/J crossed with B6.129X1-*Gt(ROSA)26Sor*$^{tm1(EYFP)Cos}$/J	Jackson Laboratory Stock 020940 and 006148	III
Csf1r$^{Mer\text{-}iCre\text{-}Mer}$; *R26R-EYFP*	FVB-Tg(Csf1r-cre/Esr1*)1Jwp/J crossed with B6.129X1-*Gt(ROSA)26Sor*$^{tm1(EYFP)Cos}$/J	Jackson Laboratory Stock 019098 and 006148	III
Ccr2^{CreERT}; *R26-TdTomato*	C57BL/6NTac-Ccr2tm2982(T2A-Cre7ESR1-T2A-mKate2)^{BB} crossed with B6.Cg-Gt(ROSA)26$^{Sortm14(CAG-tdTomato)Hze}$/J	(Croxford et al. 2015) Gift from Professor Burkhard Becher (University of Zurich) Jackson Laboratory Stock 007914	III

4.2 In vivo treatments

4.2.1 Depletion of immune cells (I, III)

4.2.1.1 Prenatal depletion of yolk sac-derived macrophages

Pregnant WT or *Plvap*$^{+/-}$ females were administered i.p. with 3 mg of CSF1R blocking antibody (clone AFS98, BE0213, Bio X Cell) or IgG2a isotype control (clone 2A3, BE0089, Bio X Cell) at E6.5 to deplete yolk sac-derived macrophages in the offspring as described (Squarzoni et al. 2014).

4.2.1.2 Postnatal depletion of macrophages

Newborn (postnatal days 0–2) WT or *Ccr2*$^{-/-}$ mice were administered i.p. with 150 µg of CSF1 neutralizing antibody (clone 5A1, BE0204, Bio X Cell) or IgG1 isotype control (clone HRPN, BE0088, Bio X Cell) to systemically deplete embryonically derived macrophages.

For prolonged systemic depletion of resident macrophages and blood monocytes, two-week old WT mice were treated with the CSF1 neutralizing antibody or IgG1 isotype control and clodronate-loaded liposomes or empty liposomes for ten days as scheduled in **Table 2**. Tissues were harvested for analyses after 40 hours, 11 days, or three weeks from the last clodronate administration.

To deplete the macrophages in the early ontogeny and to keep the macrophage deficient status through the first weeks of life, the CSF1R antibody and CSF1 antibody treatments were combined. In detail, WT pregnant mice were administered i.p. with 3 mg of CSF1R antibody at E6.5 to deplete yolk sac-derived macrophages. Subsequently, the offspring were treated with 150 µg of CSF1 antibody right after birth and again with 500 µg of CSF1 antibody at the age of two weeks.

Table 2. Schedule for prolonged macrophage and monocyte depletion.

TREATMENT SCHEDULE	AGE OF MICE (± 1 D)	TREATMENT AND ROUTE OF ADMINISTRATION
Day 1	P14	500 µg of anti-CSF1 or IgG1 isotype control, i.p.
Day 2	P15	50 µl of clodronate loaded or empty liposomes, i.v.
Day 5	P18	250 µg of anti-CSF1 or IgG1 isotype control, i.p.
Day 6	P19	50 µl of clodronate loaded or empty liposomes, i.v.
Day 9	P22	250 µg of anti-CSF1 or IgG1 isotype control, i.p.
Day 10	P23	50 µl of clodronate loaded or empty liposomes, i.v.

4.2.1 Phagocytosis assays (II, III)

Scavenging abilities of macrophage and endothelial cell populations were studied by in vivo administration of fluorescently labelled particles or macromolecules. lmmune complexes (OVA-IC) of ovalbumin (OVA)- Atto488 (41235, Sigma–Aldrich) and rabbit polyclonal OVA IgG (C6534, Sigma–Aldrich) were constituted in vitro with a 5:1 molar ratio. Mice were administered i.v. with 120 µg of OVA-IC, 10 µg of fluorescently labelled acetylated low-density lipoprotein (LDL; Alexa Fluor 488 or Alexa Fluor 594 conjugated; L23380 or L35353, Thermo Fisher Scientific), 20 µl of 0.5 µm or 0.02µm carboxylate modified microspheres (505/515 or 660/680; F8813 or F8783, Thermo Fisher Scientific) or 0.8 mg of 500 kDa Dextran (fluorescein-conjugated; D7136, Thermo Fisher Scientific) diluted in 150 µl of PBS. Control mice received 150 µl PBS. The mice were euthanized after 1 hour (Dextran) or 2 hours (others) and tissues were collected and processed for flow cytometry and imaging.

4.2.2 Cell proliferation assay (III)

WT males or pregnant dams at E17.5 were injected i.p. with 120 µl of 10 mg/ml 5-bromo-2´-deoxyuridine (BrdU, BD), that incorporates into nuclear DNA during replication in the S-phase and can be probed by specific antibody. Mice were euthanized 2 hours later to study the in vivo proliferation within macrophage populations. Tissues were processed and stained for flow cytometry with additional

intracellular staining with FITC conjugated anti-BrdU antibody kit (559619, BD) according to the manufacturer's protocol and analysed with flow cytometry.

4.3 In vitro experiments

4.3.1 Phagocytosis assays (III)

Testes from 5-week-old WT mice were dissected, homogenized and digested with 50 µg/ml DNase 1 (10104159001, Roche) and 1 mg/ml Collagenase D (1108886601, Roche) at 37 °C for 45 minutes. $CD11b^+$ cells were isolated using CD11b MicroBeads system (130-049-601, Miltenyi Biotec) according to the manufacturer's instructions. 3.0×10^5 cells were seeded to 96-well-plate wells with 100 µl of 0.5 mg/ml FITC conjugated 500 kDa Dextran diluted in RPMI-1640 medium (R5886, SAFC) supplemented with 10% foetal calf serum (FCS) and 2 mM L-glutamine. Cells were maintained at 37 °C or 4 °C for one hour, washed with PBS and stained for flow cytometry. Mean fluorescence intensity was determined and normalized to 4 °C control.

4.3.2 Colony-forming unit-culture (I)

WT and *Plvap*$^{-/-}$ tissues were harvested at indicated ages and processed to single-cell suspensions. $5\text{-}10 \times 10^3$ cells were seeded in 1 ml of M3434 Methocult medium (Stemcell Technologies) into cell culture dishes (Ø 35-mm) and maintained at 37 °C with 5% CO_2. After 7-14 days colonies were counted.

4.4 Cytometry

4.4.1 Preparation of single-cell suspensions (I-III)

Pregnant females were sacrificed from E10.5 to E17.5 by CO_2 asphyxiation and cervical dislocation. Embryos were sacrificed by decapitation. Newborn and one-week old mice were euthanized by decapitation. Mice older than that were sacrificed by CO_2 asphyxiation with subsequent cervical dislocation or exsanguination by cardiac puncture for terminal blood.

Majority of the tissue samples were dissociated mechanically to HBSS and enzymatically digested with 50 µg/ml DNase 1 (10104159001, Roche) and 1 mg/ml Collagenase D (1108886601, Roche) at 37 °C for 15-60 minutes. Alternatively, they were dispersed mechanically to flow-cytometry buffer (2% FCS (v/v) and 0.05% NaN3 (v/v) in phosphate-buffered saline (PBS)). Cells were then filtered through

silk cloth (pore size: 77 µm) washed in PBS. Microglia from enzyme digested brain were isolated through discontinuous Percoll® density gradient centrifugation (Ginhoux et al. 2010). Postnatal spleens were dissociated mechanically to PBS. Dissociated spleens and anticoagulated blood samples were lysed of erythrocytes with hypotonic saline (0.2% NaCl). Peritoneal cells were flushed with RPMI-1640 supplemented with heparin and 2% foetal bovine serum. Postnatal or embryonic femora were crushed with pestle and mortar or minced, respectively, in HBSS. Bone marrow was then filtered and washed in PBS. Postnatal livers were dissociated with GentleMACS Dissociator (Miltenyi Biotec) for one minute in RPMI-1640 medium and leukocytes were isolated by Optiprep density gradient centrifugation (D1556, Sigma). Postnatal kidneys were processed as described for postnatal livers or they were mechanically dissociated in HBSS followed by enzyme digestion.

4.4.2 Flow cytometry and fluorescence-activated cell sorting (I-III)

Prior to antibody staining, cells were stained with Fixable Viability Dye eFluor® 780 (65-0865-14, eBioscience) to exclude dead cells and subsequently blocked with anti-CD16/CD32 antibody to reduce unspecific binding of IgG to the low-affinity Fc-receptors. For surface marker labelling the cells were then incubated with antibody mixture diluted in flow-cytometry buffer at 4 °C for 30 minutes and washed with flow cytometry buffer. To label intracellular antigens, eBioscience™ Foxp3/ Transcription Factor Staining Buffer Set (00-5523-00, Invitrogen) was used according to the manufacturer's protocol. Antibodies were either directly conjugated with fluorophores or indirectly recognized with appropriate fluorophore-conjugated secondary antibodies. Antibodies used in the three studies are listed in **Table 3**. Optionally, the samples were fixed with 1% formaldehyde in PBS.

Cells were acquired on LSRFortessa flow cytometer (BD) (I-III) or acquired and collected on FACSAriaII cell sorter (BD) (I, II). Data were analysed with the FlowJo software (TreeStar Inc/FlowJo LLC).

Table 3. List of antibodies used in single-cell staining for flow cytometry and fluorescence activated cell sorting.

ANTIBODY	CLONE	COMPANY	CAT. NO.	STUDY
CD16/CD32	2.4G2	BD	553142	I
CD16/CD32	2.4G2	Bio X Cell	CUSTOM24G2	ÍI-III
B220-BV421	RA3-6B2	BD	562922	I
B220-Pacific Blue	RA3-6B2	BD	558108	I
B220-PE	RA3-6B2	BD	553089	I
CD4-A647	RM4-5	BD	557681	I

ANTIBODY	CLONE	COMPANY	CAT. NO.	STUDY
CD4-APC-Cy7	GK1.5	BD	552051	I
CD4-PE	RM4-5	BD	553048	I
CD8-BV650	53-6.7	BD	563234	I
CD8-PE	53-6.7	BD	553032	I
CD8-PerCP Cy5.5	53-6.7	BD	551162	I
CD11b-APC-Cy7	M1/70	BD	561039	I, III
CD11b-BB515	M1/70	BD	564454	I, III
CD11b-BV786	M1/70	BD	740861	II, III
CD11b-PE	M1/70	BD	553311	I, III
CD11c-BV421	HL3	BD	562782	I
CD11c-BV711	N418	BioLegend	117349	I, III
CD11c-PerCP Cy5.5	HL3	BD	560584	I
CD34-PerCP Cy5.5	HM34	BioLegend	128607	I
CD41-FITC	MWReg30	BD	553848	I
CD41-PE	MWReg30	BD	561850	I
CD45-APC-Cy7	30-F11	BD	557659	I
CD45-PerCP Cy5.5	30-F11	BD	550994	I-III
CD45-V500	30-F11	BD	561487	I, II
CD64-PE	X54-5/7.1	BioLegend	139304	I, III
CD115-PE-Cy7	AFS98	eBiosciences	25-1152-80	I, III
CD117-BV510	ACK2	BioLegend	135119	I
CD127-PE-Cy7	SB/199	BD	560733	I
CD135-BV421	A2F10.1	BD	562898	I
CD144-A647	11D4.1	BD	562242	II
CD206-A488	MR5D3	BioRad	MCA2235A488	I
CD206-BV650	C068C2	BioLegend	141723	I, III
CD304-PE	3DS304M	eBiosciences	12-3041-82	I
F4/80-A488	BM8	eBiosciences	53-4801-82	III
F4/80-A647	Cl:A3-1	Bio-Rad	MCA497A647	I-III
F4/80-PE	Cl:A3-1	Abcam	Ab105156	I
Lin-APC	145-2C11, M1/70, RA3-6B2, TER-119, RB3-8C5	BD	558074	I
Ly6C-BV421	AL-21	BD	562727	I, III

ANTIBODY	CLONE	COMPANY	CAT. NO.	STUDY
Ly6C-FITC	AL-21	BD	553104	I
Ly6G-BV510	1A8	BioLegend	127633	I, III
LYVE-1-PE	223322	R&D	FAB2125P	II
MerTK-PE	2B10C42	BioLegend	151505	III
MHC II-A488	M5/114.15.2	BD	562352	III
MHC II-BV711	M5/114.15.2	BD	563414	III
MHC II-PE	M5/114.15.2	BD	557000	III
MHCII-PE-Cy7	M5/114.15.2	eBiosciences	25-5321-82	I
PLVAP-A488	MECA-32	BioLegend	120506	II
Podoplanin-PE-Cy7	8.1.1	BioLegend	127412	II
Sca-1-PE	177228	R&D Systems	FAB1226P	I
Siglec-F-PE-CF594	E50-2440	BD	562757	I, III
VEGFR1-PE	141522	R&D Systems	FAB4711P	I
VEGFR2-PE	Avas12a1	eBiosciences	12-5821-81	I
Lineage isotype control cocktail APC	-	BD	558074	I
Rat IgG1-FITC	-	BD	553924	I
Rat IgG2a-A488	-	BioRad	MCA1212A488	I
Rat IgG2a-A647	-	BD	557690	I
Rat IgG2a-BV421	-	BD	562602	I
Rat IgG2a-BV510	-	BD	562952	I
Rat IgG2a-BV650	-	BD	56326	I
Rat IgG2a-Pacific Blue	-	BD	558109	I
Rat IgG2a-PE-CF594	-	BD	562302	I
Rat IgG2a-PE-Cy7	-	eBiosciences	25-4321-81	I
Rat IgG2a-PerCP Cy5.5	-	BD	550765	I
Rat IgG2b-A647	-	BioLegend	400626	I
Rat IgG2b-APC-Cy7	-	BD	552773	I
Rat IgG2b-BB515	-	BD	564421	I
Rat IgG2b-BV510	-	BioLegend	135119	I
Rat IgG2b-PE-Cy7	-	eBiosciences	25-4031-81	I
Rat IgG2b-PerCP Cy5.5	-	BD	550764	I
Rat IgG2b-V500	-	BD	560784	I

ANTIBODY	CLONE	COMPANY	CAT. NO.	STUDY
Rat IgM-FITC	-	BD	553942	I

4.4.3 Mass cytometry (III)

Before surface antigen staining the cells were labelled with 2.5 μM Cell-ID Cisplatin (Sigma–Aldrich, cat. 479306-1 G) to identify dead cells and blocked with CD16/32 antibody. Then the cells were stained with a heavy-metal isotope-labelled mAb mixture at RT for 30 minutes. Antibodies used for the staining are listed in **Table 4**. For single event identification, cells were treated with DNA intercalation solution (Cell ID Intercalator-103Rh in MaxPar® Fix and Perm Buffer; Fluidigm, cat. 201103 A and 201067, respectively), fixed in 4% PFA (Santa Cruz Biotechnology, cat. sc-281692) overnight and resuspended to MaxPar Water (Fluidigm cat. 201069). Data were acquired on a Helios® cytometry by time of flight (CyTOF) mass cytometer (Fluidigm) and the data were analysed in The Cytobank Solutions platform (Fluidigm).

ViSNE and FlowSOM algorithms (Cytobank) were employed to reconstruct the T-distributed Stochastic Neighbor Embedding (t-SNE) plot and to perform unsupervised subclustering of the data, respectively. X-shift algorithm of VorteX clustering and visualization environment (version VorteX 29-Jun-2017-rev2) was used to carry out unsupervised hierarchical clustering. The default settings were used with the nearest density estimation (K) from 150 to 10, with 30 steps. The force-directed layout was created from the clusters with ForceAtlas2 algorithm (all cell events from clusters smaller than 1000 events and 1000 randomly selected events from the clusters bigger than 1000 events). Clusters were analysed for the cell-type-selective differentiation markers and assigned manually to leukocyte subpopulations. The data were visualised in Gephi 0.9.1 (https://gephi.org).

Table 4. Antibodies used in staining for mass cytometry.

ANTIBODY	CLONE	COMPANY	CAT. NO.
B220-160Gd	RA3-6B2	Fluidigm	3160012C
B220-159Tb	RA3-6B2	DVS Sciences	3159015C
CD4-172Yb	RM4-5	Fluidigm	3172003C
CD8a-168Er	53-6.7	Fluidigm	3168003C
CD11b-148Nd	M1/70	Fluidigm	3148003C

ANTIBODY	CLONE	COMPANY	CAT. NO.
CD11c-142Nd	M1/70	Fluidigm	3142003C
CD45-147Sm	30-F11	Fluidigm	3147003C
CD64-151Eu	X54-5/7.1	Fluidigm	3151012C
CD68-FITC	FA-11	BioLegend	137005
CD117-173Yb	2B8	Fluidigm	3173004C
CD206-169Tm	C068C2	Fluidigm	3169021C
CD274-153Eu	10F.9G2	Fluidigm	3153016C
CX3CR1-164Dy	SA011F11	Fluidigm	3164023C
F4/80-159Tb	BM8	Fluidigm	3159009C
F4/80-146Nd	BM8	Fluidigm	3146008C
Ki-67-161Dy	B56	Fluidigm	3161007C
Ly6C-150Nd	HK1.4	Fluidigm	3150010C
Ly6C-162Dy	HK1.4	Fluidigm	3162014C
Ly6G-141Pr	1A8	Fluidigm	3141008C
LYVE-1-PE	223322	R&D Systems	FAB2125P
MerTK-FITC	2B10C42	BioLegend	151503
MHC II-174Yb	M5/114.15.2	Fluidigm	3174003C
Siglec-1-170Er	3D6.112	Fluidigm	3170018C
Siglec-F-APC	S17007L	BioLegend	155507
Siglec-F-PE-CF594	E50-2440	BD	562757
TER-119-154Sm	TER-119	Fluidigm	3154005C
Tim-3-162Dy	RMT3-23	Fluidigm	3162029C
APC-176Yb	APC003	Fluidigm	3176007C
FITC-144Nd	FIT-22	Fluidigm	3144006C
PE-165HO	PE001	Fluidigm	3165015C

4.5 Histology (I-III)

To visualize gross tissue histology, tissue sections were passed through classical histological staining. In brief, tissue samples were fixed in 4% PFA, 10% formalin, or Bouin's solution (HT10132, Sigma–Aldrich) for 2 hours at room temperature or

overnight at 4 °C. Samples were rinsed in PBS, dehydrated in an increasing ethanol series, and embedded in paraffin wax. Tissue blocks were cut with microtome to 4 µm thick sections. After deparaffinization, sections were stained with haematoxylin and eosin or periodic acid-Schiff (PAS), dehydrated, and mounted with DPX Mountant (Sigma).

Immunohistochemical staining was performed on foetal liver samples. Deparaffinised formalin fixed sections were immersed in EDTA buffer (S2367, Dako) and heated for antigen retrieval followed by an endogenous peroxide quenching with 3% H_2O_2. Rabbit serum and DakoCytomation Biotin blocking system (X0590, Dako) were used to reduce the unspecific binding of immunoglobulins and nonspecific staining of endogenous biotin. The sections were stained first with primary antibody and then with appropriate biotin conjugated secondary antibody. Biotin was recognized by using a Vectastain ABC kit (PK-6100, Vector Laboratories) following manufacturer's instructions. The signal was visualized using Liquid DAB+ substrate Chromogen System (K3468, Dako). Finally, sections were counterstained for haematoxylin, dehydrated, and mounted. All samples were imaged using Pannoramic 250 Flash II slide scanner (3D Histech Ltd.).

4.5.1 Iron recycling (I)

Prussian blue staining was used to identify ferric iron in deparaffinised PFA fixed liver and spleen sections (Haldar et al. 2014). The sections were counterstained for nuclear Fast Red (N3020, Sigma-Aldrich), dehydrated, and mounted. Images were acquired on a Panoramic 250 Flash II slide-scanner (3D Histech). The thresholding tool of ImageJ software was employed to segment and measure the areas with the blue-stained Fe^{3+}-carrying cells in the liver sections and in the red pulp area of the spleen sections.

4.5.2 Mammary gland ductal branching (I)

The abdominal mammary gland was fixed on a microscopy slide with Carnoy's medium (60% ethanol, 30% chloroform, and 10% acetic acid) at 4 °C overnight. Rehydrated samples were stained with carmine alum (0.2% carmine, 0.5% aluminium potassium sulfate dodecahydrate) at room temperature overnight, dehydrated, cleared with xylene, and mounted in DPX Mountant (Sigma). Images were acquired on a Zeiss SteREO Lumar V12 stereo microscope equipped with NeoLumar 0.8× objective and Zeiss AxioCam ICc3 colour camera. The number of ductal branches was quantified by the Skeletonize2D/3D and AnalyzeSkeleton plugins of ImageJ software from the manually chosen area covered by the ducts.

4.5.3 Cytology (I)

Sorted macrophages from the foetal liver were collected with Shandon Cytospin 3 centrifuge (Thermo) onto microscope slides. Samples were then stained with Diff-Quick (REASTAIN) and mounted in DPX Mountant (Sigma). The cytospun slides were imaged using a Zeiss AxioVert 200M (Zeiss) equipped with a Plan-Neofluar 40×/0.60 NA objective.

To determine the leukocyte content from the peripheral blood of adult *Plvap*$^{-/-}$ and WT mice, heparinized blood was analysed with Automated hemocytometer (VetSCan HM 5, Abaxis). Additionally, concentrations of total protein, albumin, amylase, and urea nitrogen from the plasma samples were analysed with VetScan VS2 (Abaxis).

4.5.4 Detection of luminal PLVAP (I, II)

WT embryos in their yolk sacs were dissected out of the uterus. Foetal liver sinusoidal PLVAP was labelled by injecting unconjugated monoclonal anti-PLVAP (MECA32, custom-made, InVivo BioTech) or isotype-matched control antibody (rat IgG2a 553926, BD) to the umbilical or vitelline veins of the yolk sac. After one-minute circulation time, embryos were euthanized, and livers were harvested and processed for imaging (I). Alternatively, the antibodies were administered via tail vein injection into 4-week-old WT or *Plvap*$^{-/-}$ mice. For immunoelectron microscopy, anti-PLVAP antibody was directly conjugated with gold nanoparticles using a GOLD conjugation kit (201808, Abcam) according to the manufacturer's protocol prior to administration. After 10 minutes circulation time, mice were euthanized and livers were excised and processed for imaging or transmission electron microscopy (II).

4.5.5 Immunofluorescence and imaging (I-III)

For immunofluorescence, either fresh-frozen or 4% PFA fixed paraffin sections were used. Prior to cryosectioning, tissues were embedded in an optimal cutting temperature (OCT) compound (4583, Tissue-Tek) and frozen with either dry ice or liquid nitrogen. Samples were cut into 5-6 µm thick sections with cryostat and immediately fixed with ice-cold acetone or 95% ethanol. The samples were sequentially incubated with primary antibodies, secondary antibodies, and directly conjugated antibodies diluted in PBS containing 5% serum and 0.1% Triton X-100 (T8787, Sigma-Aldrich). All the antibodies used for any of the imaging techniques are listed in **Table 5**. When indicated, the samples were counterstained with Hoechst (62249, Thermo Fisher Scientific). The sections were mounted with ProLong Gold

Antifade Mountant with or without DAPI (4,6-diaminodino-2-phenylindole; ThermoFisher Scientific, #P36931 or #P36930).

Before immunostaining, PFA fixed and deparaffinised tissue sections were incubated in 0.1 M citrate buffer, pH 6 at 95 °C for antigen retrieval and in 0.1 M NH_4Cl to reduce autofluorescence. 5% serum was used to decrease unspecific binding. Samples were then incubated with primary antibodies followed by appropriate fluorophore conjugated secondary IgG and, finally, mounted in SlowFade™ Diamond Antifade mounting medium with DAPI (S36964, Thermo Fisher Scientific). Images from the paraffin and frozen sections were acquired on a Pannoramic Midi fluorescence slide scanner (3DHISTECH Ltd) and analysed with CaseViewer 2.4 software. Alternatively, an LSM 780 or LSM 880 confocal microscope (Carl Zeiss) equipped with Plan-Apochromat 20×/0.8, c-Apochromat 40x/1.20 or c-Apochromat 63x/1.20 objective was used with Zen 2010 software (Zeiss) to capture images.

Table 5. Antibodies used in imaging and protein probing.

ANTIBODY	CLONE	COMPANY	CAT. NO.	STUDY
AlphaSMA-A488	1A4	Abcam	ab184675	III
Caveolin	pAb	Santa-Cruz	sc-894	I
CD31	MEC 13.3	BD	550274	I
CD31	MEC 13.3	BD	553370	III
CD31-APC	MEC13.3	BioLegend	102510	I, II
CD117 (c-kit)	2B8	BD	553352	I
CD144-A647	11D4.1	BD	562242	II
CD206-A488	MR5D3	BioRad	MCA2235A488T	III
CD206-A647	MR5D3	BD Biosciences	565250	III
F4/80	CI:A3-1	Bio-Rad	MCA497	II, III
F4/80	CI:A3-1	Bio X Cell	BE0206	III
F4/80-A488	BM8	Invitrogen	MF48020	I
GFP	GF28R	Invitrogen	A11122	I
Hsd3b	pAb	TransGenic Inc	KO607	III
Ki-67	SolA15	eBioscience	11-5698-82	III
LYVE-1	pAb	Reliatech	102-PA50AG/103-PA50	I, II
MAdCAM-1	MECA-367	E.Butcher Stanford Univ.	-	I

ANTIBODY	CLONE	COMPANY	CAT. NO.	STUDY
MHC II-A647	M5/114.15.2	BioLegend	107618	III
Neuropilin-1	pAb	R&D	AF566	I, II
PLVAP	PAL-E	Abcam	Ab8086	I
PLVAP	Meca-32	Invivo	MECA32-17-8 AK 2028/01	I
PLVAP	Meca-32	Bio X Cell	BE0200	II
PLVAP	Meca-32	BD	550563	I, II
PLVAP	PAL-E	Abcam	ab8086	I
VEGF-A	pAb	Abcam	Ab46154	I
VEGF-A	A-20	Santa Cruz	sc-152	I
VEGFR2	pAb	R&D	AF644-SP	II
CLDN11	H-107	Santa Cruz	sc-25711	III
SOX9	pAb	EMD Millipore	AB5535	III
Espin	31/Espin	BD Biosciences	611656	III
Anti-goat IgG-A647	pAb	Invitrogen	A21447	II
Anti-mouse IgG-A488	pAb	Invitrogen	A11029	I
Anti-rabbit IgG-A546	pAb	Invitrogen	A11035	I-III
Anti-rabbit IgG-A594	pAb	Invitrogen	A21207	II
Anti-rabbit IgG-A633	pAb	Invitrogen	A21071	I
Anti-rat IgG-A488	pAb	Invitrogen	A11006	II, III
Anti-rat IgG-A488	pAb	Invitrogen	A21208	I, II
Anti-rat IgG-A546	pAb	Invitrogen	A11081	I-III
Anti-rat IgG-A594	pAb	Invitrogen	A11007	III
Anti-rat IgG-A647	pAb	Invitrogen	A21247	I, II
Anti-rat IgG-gold	pAb	BBI Solutions	EM.GAT10	II
Rat IgG2a	R35-95	BD	553926	II

4.5.5.1 Whole-mount (I-III)

Tissues were harvested and prepared for whole-mount staining as previously described (Yokomizo et al. 2012). Briefly, tissues samples were fixed in 2% PFA in

PBS on ice for 20-60 minutes, washed in PBS, and dehydrated in an increasing methanol series. Samples were kept in 100% methanol at −20 °C overnight, rehydrated in a decreasing methanol series and blocked with 1% normal mouse, rabbit, or rat serum and 0.5% FCS in a washing buffer (PBS containing 1% skim milk or 1% bovine serum albumin (BSA) and 0.4% Triton X-100). Samples were then incubated overnight with the primary antibodies or isotype-matched IgG control antibodies in the washing buffer. After washing extensively in the washing buffer, samples were incubated overnight with secondary antibodies diluted in the washing buffer supplemented with 5% normal mouse, donkey, rat, or rabbit serum. After washing extensively in the washing buffer and rinsing in 0.4% Triton X-100–PBS, samples were dehydrated and optically cleared with BABB (mixture of benzyl alcohol 1:2 benzyl benzoate; 402834, Honeywell and B6630 Sigma–Aldrich, respectively) on glass bottom microwell dishes (MatTek). Finally, coverslips were mounted on the microwells. For certain samples Hoechst was added to the final wash with the washing buffer prior to rinsing to counterstain nuclei.

For seminiferous tubule whole-mount staining, the tunica albuginea of testis was removed. Samples were prepared for whole-mount staining as previously described (Faisal et al. 2019). Briefly, nonspecific binding was blocked with 2% BSA and 10% foetal bovine serum diluted in 0.3% Triton X-100–PBS followed by sequential incubation with unconjugated primary antibodies, fluorophore conjugated secondary antibodies, and directly conjugated antibodies all diluted in appropriate blocking solutions. The seminiferous tubule samples were placed on linear strips and mounted with SlowFade™ Diamond Antifade mounting medium with DAPI.

Samples were imaged with a spinning disk confocal microscope (Intelligent Imaging Innovations) equipped with Orca-Flash4 v2 sCMOS camera (Hamamatsu) and Plan-Apochromat ×20/0.8 or C-Apochromat ×40/1.1 objective and using SlideBook 6 software (Intelligent Imaging Innovations) or a LSM780 confocal laser-scanning microscope (Carl Zeiss) equipped with Plan-Apochromat ×20/0.8 or C-Apochromat ×40/1.2 objective and Zen 2010 software (Zeiss).

4.5.5.2 Vibratome sections (II)

To better visualize the location of macrophages in the testes of postnatal mice, the testes preserved for whole mount staining were rehydrated and embedded in 4% low-melting agarose (50080, Lonza). Polymerized block was cut into 300 µm sections with VT1200 S vibratome (Leica). After mechanical removal of the agarose, sections were stained as described for whole-mount samples.

4.5.6 Transmission and scanning electron microscopy (I, II)

Adult mice but not embryos were perfusion fixed with 2% glutaraldehyde and 4% PFA prior to tissue harvesting. Livers were fixed in 5% glutaraldehyde in 0.16 M s-collidine buffer for transmission electron microscopy (TEM) or in 2% glutaraldehyde and 4% PFA for scanning electron microscopy (SEM), post-fixed with 2% OsO_4 containing 3% potassium ferrocyanide for 2 hours and dehydrated in an increasing ethanol series.

Samples for TEM were then embedded in epoxy medium (kit no. 45359, Fluka). 70 or 150 nm sections were cut with an ultramicrotome and stained with 1% uranyl acetate and 0.3% lead citrate. Samples were examined in a JEM-1400 Plus transmission electron microscope. Samples for SEM were snap frozen in liquid nitrogen, manually fractured, and dried with hexamethyldisilazane. Liver pieces were then coated with gold, and images were acquired on Leo 1530 Gemini scanning electron microscope. ImageJ software was used to measure the surface areas of vessels and the fenestral openings.

4.5.7 Immunoelectron microscopy (II)

Mice were perfusion fixed with 0.2% glutaraldehyde in PLP-fixative (0.01 M periodate, 0.075 M lysine and 2% formaldehyde in $NaPO_4$, pH 7.4). The excised livers were immersed in the same fixative for 1 hour at RT, cryoprotected with 2.1 M sucrose overnight at 4 °C, embedded in OCT, and frozen in liquid nitrogen. 10 µm sections were blocked for unspecific binding of IgG with 1% fish gelatin (G-7765, Sigma) and 1% BSA in 0.1 M $NaPO_4$ followed by consecutive incubation with primary and gold-conjugated secondary antibodies diluted in 0.5% fish gelatin and 0.5% BSA in 0.1 M $NaPO_4$. After washing, the sections were post-fixed first with 2% glutaraldehyde and then with 1% osmium tetroxide, both in 0.1 $NaPO_4$. Samples were dehydrated first in increasing ethanol series and then in acetone before embedding in Epon resin. Samples were cut into 60 nm sections with EM Ultracut UC6i ultramicrotome (Leica Microsystems GmbH) and stained with uranyl acetate and lead citrate. Electron tomography was performed on a JEM-1400 Plus transmission electron microscope (JEOL).

4.5.8 Image analysis (I-III)

ImageJ software (http:// rsb.info.nih.gov/ij) was used for all the image analyses performed in the studies of this thesis, unless mentioned otherwise. Maximum intensity projections of Z-stacks acquired from whole mount staining and vibratome section staining were produced from selected optical slices prior to image processing. A background subtraction with a rolling ball algorithm was performed

after optional noise reduction with light median or mean filtering for the images. Brightness and contrast were linearly adjusted if needed. All the adjustments were equally performed to all the images compared. To produce videos of the optically sliced samples, the Z-stacks were reconstructed into three-dimensional images and converted to video format (AVI or QuickTime) with Imaris 8.0 (Bitplane).

To analyse the macrophage content in the red pulp area of spleen from *Plvap*$^{-/-}$ and WT mice, F4/80^{high} cells were highlighted by thresholding and quantified (I). MadCAM-1 staining in the marginal zone was used as guidance for white pulp area exclusion, and the area fraction occupied by F4/80^{high} cells in the red pulp was measured. A spleen area of at least 2.1 mm^2 per mouse was analysed.

To determine the relative expression intensity of PLVAP in the vascular endothelium of different zonal segments in the liver, original images of immunofluorescence staining were thresholded and selected by their signal for PLVAP and CD31. Mean grey values were calculated from the selected areas and normalised to the value calculated for portal vein. Area of signal measurements for LYVE-1 and PLVAP signals were also done using imageJ from Li, Otsu, or Moments thresholded selections. The image processing was applied equally to all images compared with each other.

Vessel diameters were measured from manually chosen LYVE-1$^+$ liver sinusoids. 30 vessels per genotype were analysed from two WT and two *Plvap*$^{-/-}$ mice.

The number of SOX9$^+$ Sertoli cells was counted from a minimum of 30 rounded seminiferous tubule cross-sections in each testicular section (II). The number of 3βHSD$^+$ Leydig cells was calculated in a measured testicular area (II). To measure the diameter of the seminiferous tubules only those with circular cross-sections were included (II). Then the diameter was determined as an average of two perpendicular measurements with Pannoramic viewer 1.15 software (3DHISTECH Ltd). A minimum of 30 tubule cross-sections from different regions of the testis were analysed.

Z-stacks acquired from vibratome sections were analysed for the blood vessel-associations with testicular macrophages (II). The CD206$^+$ or MHC II$^+$ cells were manually assigned to have or not to have direct contact with CD31$^+$ vessels.

4.6 Gene expression studies (I, II)

4.6.1 Quantitative real-time PCR (I, II)

Total RNA was extracted from the foetal livers using the Nucleo-Spin RNA kit (740955.50, Macherey–Nagel) (I). RNAeasy Plus Micro kit (Qiagen) was used to extract RNA from the sorted EMP and macrophages (I) or the sorted liver endothelial

cells (II). Reverse transcription of the RNA to complementary DNA was carried out using the SuperScript VILO cDNA Synthesis kit (Thermo Fisher Scientific) (I) according to the manufacturer's instructions. Studied genes with their primer sequences or access IDs are listed in **Table 6**. Quantitative real-time PCR (RT-qPCR) was performed using Taqman Gene Expression Assays (Thermo Fisher Scientific) and the reactions were carried out on a 7900HT Fast Real-Time PCR System (Applied Biosystems/ Thermo Fisher Scientific) or a QuantStudio® 12K Flex Real-Time PCR System (Thermo Fisher Scientific) at the Finnish Functional Genomics Centre (formerly the Finnish Microarray and Sequencing Centre), Turku Bioscience Centre, Turku, Finland. Relative mRNA expression was calculated using Sequence Detection System Software v2.4.1, QuantStudio 12 K Flex software, and DataAssist software (I) (all from Applied Biosystems/ThermoFisher Scientific). Relative mRNA expression level was determined by normalizing the target gene expression to control gene (*Actb*) expression.

Table 6. Primers used for quantitative real-time PCR.

GENE	DESCRIPTION (PRIMER OR SEQUENCE)	STUDY
Actb	Mm00607939_s1	I, II
Adgre1/Emr1/F4/80	Mm00802529_m1	I
Itgam	Mm00434455_m1	I
Gata2	Mm00492301_m1	I
Flt3	Mm00439016_m1	I
Ccr2	Mm04207877_m1	I
Mrc1	Mm00485148_m1	I
Cx3cr1	Mm00438354_m1	I
Plvap	Mm00453379_m1	I, II
Lyve-1	Mm00475056_m1	I, II

4.7 Protein interaction studies (I)

Antibodies used in protein interaction studies are listed in **Table 5**.

4.7.1 Generation of PLVAP-Fc fusion protein (I)

Full-length complementary DNA of mouse PLVAP (MR206983, Origene) was used as a template to amplify the extracellular domain (residues 48–438) of the protein by

PCR. The reaction was performed using Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific) with primers introducing EcoRV and NheI restriction sites. After purification the amplified fragment was ligated to pFUSEN-hG2Fc vector (InvivoGen). The construct was validated for integrity by sequencing and the yielded protein product for reactivity to anti-MECA-32 antibody by immunoblotting. Lipofectamine (Invitrogen) was used to transfect the expression plasmid with PLVAP-ECD-Fc or a CD4-Fc chimaera, as control, into HEK293-EBNA cells (CRL-10852, ATCC). The cells were then cultured for 2–3 days in a serum-free medium (Pro293A-CDM, Bio-Whittaker).

4.7.2 Heparin-affinity pull-down assay (I)

Total protein lysates from E14.5 WT livers or the cells transfected with the expression plasmids were obtained by lysing the tissue or cells in Tris-buffered saline (TBS; pH 7.2) containing 0.5% NP-40 and 1% BSA. Agarose beads coupled with heparin (Sigma) or streptavidin (GE Healthcare), as a negative control, were rinsed and blocked with 1% BSA in TBS. The beads were then incubated with the lysates for 2 hours at 4 °C and washed with 0.3% NP-40 in TBS. To test the affinity of the fusion protein for the beads in presence of competitive molecules, 100 µg of fibronectin (F1141, Sigma) or 100 µg of collagen (C8919, Sigma) was added to the samples. The bound molecules were eluted in Laemmli's sample buffer or alternatively for the fusion proteins in 1.0 M NaCl and resolved by SDS–PAGE. Molecules were then transferred to nitrocellulose membranes and visualized by immunoblotting with a horseradish-peroxidase-conjugated anti-human IgG antibody (Invitrogen, cat. 81-7120) or blotted with other antibodies listed in Table 5 and identified by appropriate HRP-conjugated second-stage reagents using Enhanced chemiluminescence (ECL) detection.

4.7.3 Far Western blotting (I)

The PLVAP–Fc chimaera in 5% BSA–TBS was allowed to bind to recombinant mouse neuropilin-1 (5994-N1, R&D Systems) and recombinant mouse VEGF164 (493-MV, R&D Systems) immobilized on the filters in the presence or absence of 50 IU heparin (stock 5,000 IU/ml; Leo Pharma) for 2 hours. The bound protein was detected using HRP-conjugated anti-human IgG antibody and ECL.

4.7.4 Co-immunoprecipitation assay (I)

Foetal livers of wild type mice were dissected and immediately dissociated in a lysis buffer [1% NP-40, 150 mM NaCl, 20 mM HEPES (pH 7.5), 2 mM $MgCl_2$, 2 mM

$CaCl_2$, PhosSTOP and Protease inhibitor cocktail (Roche)]. Lysates were cleared by centrifugation and probed with anti-VEGF antibody or control antibody for 5 hours at 4 °C followed by incubation with Protein G beads for 1 hour. After washing the beads with the lysis buffer, proteins were eluted in nonreducing Laemmli's sample buffer, resolved by SDS-PAGE, and immunoblotted for anti-VEGF and anti-PLVAP antibodies. Proteins were then indirectly identified by IRDye-conjugated secondary antibodies and visualized on an Odyssey imager.

4.7.5 Western blotting (II)

Livers were mechanically dissociated in a lysis buffer containing 0.2% NP-40, 150 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris, pH 7.2, and Roche Protease Inhibitor Cocktail. Lysates were cleared by centrifugation and protein concentration was determined using Bio-Rad DC kit (5000112, Bio-Rad). Proteins were denatured in nonreduced Laemmli's sample buffer at 37 °C for 10 minutes. 5 µg of protein was loaded and resolved in nonreducing SDS-PAGE, transferred to nitrocellulose membrane, and blotted with anti-PLVAP. Antibody signal was detected by HRP-conjugated secondary antibody with ECL. To probe GAPDH as control filter was then stripped and reblotted with the appropriate antibody. Mean grey values of PLVAP and GAPDH bands were determined in ImageJ. Relative expression of PLVAP was quantified by calculating the signal ratio of PLVAP to GAPDH.

4.7.6 Proximity ligation assay (I, II)

Proximity ligation assay (PLA) was used to detect associations between proteins in situ in embryonic and adult liver (Söderberg et al. 2006). Duolink in situ PLA probe anti-rabbit PLUS (DUO92002, Sigma) or anti-goat PLUS (DUO92003, Sigma) were used to probe VEGF, VEGFR2, LYVE-1 or neuropilin-1, whereas MINUS PLA probe using Duolink in situ probemaker MINUS kit (DUO92010, Sigma) was directly conjugated to antibodies against PLVAP or neuropilin-1. After ligation and amplification, with simultaneous detection of PLVAP antibody by Alexa Fluor 488-conjugated anti-rat IgG (A11035, Invitrogen), the probes were detected using Detection reagent red (DUO92008, Sigma). The samples were counterstained with DAPI and mounted in Mowiol. Images were acquired on a 3i Spinning Disk confocal microscope (Intelligent Imaging Innovations) with a plan-apochromat 63×/1.4 NA oil objective and SlideBook 6 software (Intelligent Imaging Innovations). Background subtraction with rolling ball method and linear brightness adjustments were performed equally to images taken from control and VEGF-PLVAP PLA examined samples using ImageJ.

4.8 Statistical analyses (I-III)

Data are presented as mean ± standard error of mean (SEM) or standard deviation (SD). Statistical analyses were performed in GraphPad Prism (GraphPad Software, LLC). When comparing two experimental groups, Mann-Whitney U test was employed, and for comparing data from three or more experimental groups One-Way or Two-Way analysis of variance (ANOVA) with Bonferroni or Tukey's post-hoc test was used.

5 Results

5.1 PLVAP is involved in the formation of resident macrophage populations (I)

PLVAP is a transmembrane protein best known for its expression on endothelial cells (Schlingemann et al. 1985; Stan 2007; Hallmann et al. 1995). It is the sole known component of diaphragm, a sieving structure with cartwheel resemblance, found on top of fenestrae, TEC and caveolae (Stan et al. 2012; Tkachenko et al. 2012; Bearer and Orci 1985). PLVAP is known to regulate the transmigration of macromolecules through endothelial cells (Stan et al. 2012). A previous study showing that PLVAP is involved in lymphocyte migration in the sinus-cortex interface in lymph nodes led us to examine the immune aspects of this molecule more broadly (Rantakari et al. 2015). Here, we have studied the adult *Plvap*$^{-/-}$ mice and found them to lack foetal-derived macrophages in several tissues.

5.1.1 *Plvap*$^{-/-}$ mice have less foetal-derived macrophages (I)

To study the impact of PLVAP deficiency on immune cell populations in adult mice we performed flow cytometry analyses on selected tissues from *Plvap*$^{-/-}$ mouse model. For determination of different macrophage populations we took advantage of the expression profile of F4/80, which is known to be expressed highly in foetal-derived macrophages while it is present in intermediate levels on the bone marrow monocyte-derived macrophages (Schulz et al. 2012). Interestingly, the F4/80^{high} resident macrophage populations in the spleen and peritoneal cavity were drastically diminished in *Plvap*$^{-/-}$ mice compared to WT controls. Similarly, foetal-derived alveolar macrophages with typical CD11c^{high} phenotype (Gautier et al. 2012; Guilliams et al. 2013) were lacking in the lungs of *Plvap*$^{-/-}$ mouse. (I: Fig 1a) Notably, the F4/80^{intermediate} adult bone marrow-derived macrophages were present in normal or slightly higher numbers in PLVAP-deficient mice as were the recent migrants of tissue monocytes in spleen and liver (I: Fig 1a, SFig 2a, e-f). In concordance with that, haematopoiesis in the adult bone marrow under PLVAP deficiency remained intact as indicated by WT-comparable frequencies of the HSCs, CMPs, and CLPs, including their direct descendants, bone marrow and blood

Ly6C^{high} and Ly6C^{low} monocytes and lymphocytes in various tissues studied. Moreover, *Plvap*$^{-/-}$ bone marrow cells were indistinguishable from WT control in in vitro colony forming capacity. (I: Fig 1a, SFig 2b-d & g) These results suggest that PLVAP deficiency, specifically, is linked to diminished foetal liver-derived resident macrophage populations.

5.1.2 PLVAP deficiency leads to monocyte accumulation in the foetal liver (I)

Tissue macrophages seed the tissues before birth and they can be categorized in at least two different subpopulations by their expression for F4/80 and Ly6C. In embryos, F4/80^{high} macrophages are considered to represent the yolk sac-derived primitive macrophages while F4/80^{intermediate} and Ly6C^{+} cells are thought to be differentiated from foetal monocytes (Hoeffel et al. 2015). Using these markers, we confirmed the cell type-specific morphology and the lineage-specific gene expression profile of the sorted F4/80^{high} primitive macrophages and F4/80^{intermediate} Ly6C^{+} monocyte-derived macrophages from E14.5 and E16.5 WT livers (I: SFig 5a-b). Our flow cytometry data revealed that primitive macrophage population sizes were unaffected, and the microglia that are derived from the primitive macrophages were present in normal frequencies in the embryonic (E16.5) and adult *Plvap*$^{-/-}$ brain. In contrast, the foetal liver-derived macrophages and Ly6C^{+} monocyte precursors were lacking from the spleen and lung and from the blood, respectively, of *Plvap*$^{-/-}$ mice at E16.5. (I: Fig 1b & 2a)

We then analysed the main embryonic haematopoietic sites, foetal liver, and extra embryonic yolk sac for their macrophage content and observed significant increase in F4/80^{intermediate} macrophages in foetal liver of *Plvap*$^{-/-}$ mice compared to WT control starting from E14.5. More detailed flow cytometry analysis on the foetal *Plvap*$^{-/-}$ livers revealed that both the Ly6C^{-} and Ly6C^{+} monocyte frequencies were increased significantly, whereas those of the monocyte progenitor cells, the EMPs, and HSCs were comparable to WT controls. (I: Fig 2b-d, SFig 5c-e)

Although PLVAP is expressed by endothelial cells of blood islands in the yolk sac and vascular beds in the AGM region, frequencies of primitive macrophages and EMPs (not expressing *Plvap*) in the E10.5 and E12.5 yolk sacs, and the c-Kit^{+} HSCs in the E10.5 AGM of PLVAP-deficient mice were unchanged. Also, *Plvap*$^{-/-}$ E10.5 yolk sac and E12.5 liver cells had equal colony-forming potential in vitro to WT controls. (I: Fig 2a-b, SFig 4a-g) Disturbance of the primitive macrophage development in the *Plvap*$^{-/-}$ offspring by single injection of anti-CSF1R (ASF98) antibody to pregnant dam at E6.5 had no effect on the number of foetal monocytes in the liver at E14.5 (I: SFig 6a-c). Taken together, these results indicate that PLVAP is needed for the normal emigration of monocytes from the foetal liver.

5.1.3 PLVAP can bind chemoattractants via a heparin bridge (I)

PLVAP was detected by immunohistochemistry in WT foetal liver from E11.5 onwards and respective gene expression (*Plvap*) was confirmed by RT-qPCR. As expected, neither protein nor mRNA expression of PLVAP were seen on the knockout samples (I: Fig 3a-c, SFig 7a-b). Our histological staining shows that PLVAP is exclusively expressed on the LYVE-1^+ and $CD31^+$ endothelial cells in foetal liver. Moreover, intravenous administration of PLVAP antibody recognized PLVAP on the luminal side of the liver sinusoidal endothelial cells. This specific expression was also seen in other WT mouse strains and in humans. (I: Fig 3c SFig 7c-i) Finally, timed deletion of PLVAP precisely at E12.5 in *Plvap*$^{F/F}$;*CAGGCre-ER*^{TM} mice that we generated and conditional deletion of PLVAP in Lyve-1^+ cells both led to monocyte accumulation in the liver at E14.5 (I: SFig 8a-f).

As fenestrations on the liver sinusoidal endothelial cells were left open without the PLVAP diaphragms as shown in *Plvap*$^{-/-}$ livers at E12.5, we wondered if PLVAP might bind chemoattractants which would lure and guide monocytes from the liver stroma to vasculature (I: Fig 3d). In agreement with this hypothesis we saw binding of PLVAP from E14.5 liver lysates to heparin-affinity beads. Furthermore, VEGF-A, another heparin-binding molecule, co-immunoprecipitated PLVAP via a heparin bridge, VEGF-A staining overlapped with PLVAP in in situ immunofluorescence staining, and they were within interaction range from each other as indicated by proximity ligation assay. (I: Fig 3e-f, SFig 9 a-b) In further biochemical analyses we found that PLVAP also bound via a heparin bridge to neuropilin-1, which is a potential counter-receptor for monocyte-expressed VEGFR1 (I: Fig 3g-h, SFig 9c-i). These results suggest that PLVAP may guide the exit of monocytes from the foetal liver by immobilizing chemoattractants or serving as a counter-receptor for adhesion molecules (both functions via a heparin bridge).

5.1.4 Iron recycling and ductal branching of mammary gland are impaired in adult PLVAP-deficient mice (I)

We then investigated the functional consequences of the deficient foetal liver-derived macrophages in selected tissues. Some functions like bronchial branching and interdigital regression were preserved in *Plvap*$^{-/-}$ embryos (I: SFig 10a-b). However, the lack of F4/80^{high} macrophages in the red pulp area of spleen and in the liver of PLVAP-deficient pre-pubertal mice was observed together with Fe^{3+} build up, which is in line with the fact that those macrophages are responsible for recycling the iron from senescent erythrocytes (Haldar et al. 2014) (I: Fig 4a-b, SFig 10c). Strikingly, the ductal branching in the mammary gland of pre-pubertal *Plvap*$^{-/-}$ females with significantly reduced number of F4/80^{high} macrophages was severely

diminished (I: Fig 4c-f, SFig 10d-e). In conclusion, our data suggest that PLVAP is involved in the exit of monocytes from the foetal liver affecting the generation of tissue macrophage populations. Moreover, those captive monocytes play critical roles in organ-specific key functions.

5.2 PLVAP expression and associations are altered in liver sinusoids through aging (II)

As we showed that PLVAP played a crucial role in foetal monocyte migration in the foetal liver, we were intrigued to find out what happens to PLVAP after birth, when the main haematopoietic activity gradually shifts from liver to bone marrow. This was relevant because earlier publications implied that the PLVAP constituted diaphragms are lost from the adult liver sinusoidal endothelium, while mRNA and protein expression of PLVAP remains observable (Herrnberger et al. 2014; Stan et al. 1999).

5.2.1 PLVAP expression persists in the LSECs of adult mice (II)

Fenestral diaphragms have been shown to appear in LSECs of the foetal liver at E12.5 but are absent after birth (Herrnberger et al. 2014; Wisse 1970). Accordingly, we observed the diaphragms on fenestrae in LSECs at embryonic time points (E12.5–18.5) but not after birth as visualized by transmission electron microscopy. Also, in in situ immunofluorescence staining, PLVAP expression is detected in the immediate vicinity of LYVE-1 expression, which is a known marker of LSECs (Carreira et al. 2001) (I: Fig 3d, II: Fig 1d). Interestingly, at postnatal time points we detected a similar expression pattern for PLVAP and LYVE-1 as during the foetal period. Furthermore, we saw strong PLVAP expression in the portal and central veins, but it was also evident, although less intensive, in the pericentral, periportal, and midlobular zones of the liver parenchyma (II: Fig 2b-d). Finally, we confirmed expression of *Plvap* in the LSEC of postnatal liver as determined by RT-qPCR of the sorted cells (II: Fig 3d). In conclusion, PLVAP is robustly expressed in the LSEC of adult mice.

5.2.2 Immuno-complex scavenging is interfered in adult *Plvap*$^{-/-}$ liver (II)

LSECs are known for their superior capacity to scavenge macromolecules from the bloodstream and for their role in intensive trafficking between blood and liver parenchyma (Seternes, Sørensen, and Smedsrød 2002). These functions could be

affected by the lack of fenestrations that are thought to require PLVAP to form (Herrnberger et al. 2014). In contrast, we found fenestrae comparable in numbers and size to the WT control in our *Plvap*$^{-/-}$ model at the age of 5 weeks. Of note, the vascular density and composition were indistinguishable between *Plvap*$^{-/-}$ and WT livers at E14.5 (II: Fig 5a-f). We identified a specific luminal expression of PLVAP in the LSEC in five-week-old mice as determined by the positive signal of intravenously administered MECA-32 antibody detected in the WT but not in the *Plvap*$^{-/-}$ mice (II: Fig 4a). Thus, despite the loss of diaphragm complexes, PLVAP is still present and might serve other functions in controlling permeability. We tested this possibility by injecting particles of different sizes intravenously to WT and PLVAP-deficient mice. Indeed, we observed reduced binding or uptake of ovalbumin–anti-ovalbumin immune complexes by both the F4/80^{high} macrophages, putative Kupffer cells, and the LSEC in *Plvap*$^{-/-}$ mice compared to the WT control (II: Fig 5g-i, SFig 7c-e). Taken together, our findings demonstrate that PLVAP has functions outside diaphragms.

5.2.3 PLVAP association with neuropilin-1 and VEGFR-2 is lost in adult LSECs (II)

As mentioned earlier, PLVAP is expressed on the cell surface of the LSEC in adult mice, and our earlier data demonstrated that PLVAP from the E14.5 liver binds neuropilin-1 via a heparin connector (I: Fig 3e-f, h). Together these led us to ask if PLVAP had associations with the VEGF-VEGFR-2-neuropilin system (Simons, Gordon, and Claesson-Welsh 2016). To address the question, we performed in situ proximity ligation assays, which indicate if two molecules are within 40 nm range of each other (Alam 2022), on E14.5 and adult WT livers. We found a positive signal from probing neuropilin-1 with PLVAP in the E14.5 liver. Furthermore, we observed a similar association by probing VEGFR-2, which is mainly expressed by the endothelial cells, with PLVAP but also when neuropilin-1 was probed with VEGFR-2, all in the E14.5 liver. Strikingly, these signals were lost in all the probing setups in the liver of five-week-old mice. (II: Fig 6b-d) In conclusion, protein-protein interactions of PLVAP in the LSEC change from the foetal era to adulthood.

5.3 Testicular macrophages are largely derived from foetal monocytes (III)

While studying macrophage populations in *Plvap*$^{-/-}$ mice, we also observed a slight but significant decrease in the testicular macrophage pool. Testicular macrophage populations in mice have been studied earlier (DeFalco et al. 2014; DeFalco et al.

2015; Mossadegh-Keller et al. 2017), but full consensus of their origins has not been reached.

5.3.1 Two distinct macrophage populations infiltrate embryonic testis (III)

We identified CD45^{low} F4/80^{high} and CD45^{high} F4/80^{intermediate} macrophage populations in the WT testis through flow cytometry and observed an increase in the F4/80^{intermediate} cell number leading them to represent the dominant macrophage population by birth (III: Fig 1a, SFig 2a). The F4/80^{high} putative yolk sac-derived primitive macrophages were also positive for CSF1R and CD206, whereas the F4/80^{intermediate} cells co-expressed Ly6C. Neither population showed significant expression for eosinophil marker SiglecF or dendritic cell marker CD11c. (III: Fig 1b, SFig 2 b-e) Notably, the F4/80^{high} cells were absent in the E17.5 testis after a single CSF1R antibody treatment to the pregnant dam at E6.5, which we showed to abolish the yolk sac-derived microglia from the brain (I: SFig 6b & III: Fig 1 d, SFig 2f-g).

To study the single cell surface marker profile of F4/80^{+} cells in detail, we passed pools of testes from newborn WT mice through mass cytometry analysis and t-distributed stochastic neighbour embedding (t-SNE) algorithm. We found three different macrophage populations, defined by the expression of CD64 and CX3CR1. A SiglecF^{+} eosinophil population and a few putative dendritic cells were also present, as suggested by the expression of CD11c and MHC II. The in silico lineage tracing that determines the relatedness of cells by comparing their similarity to the nearest neighbours, showed linear trajectory from Ly6C^{high} cells through Ly6C^{low}CD64^{+} intermediates and CD206$^{–}$ macrophages to CD206^{+} macrophages. (III: Fig 1 e-g, SFig 2h-i) Taken together, these results hint that the testicular macrophage pool around the time of birth is mainly derived from monocytic precursors but yet has a small contribution from primitive macrophages.

5.3.2 Testicular macrophage population shows high heterogeneity (III)

Our t-SNE analysis of the mass cytometry data acquired from the postnatal testes revealed dynamic changes in the macrophage pool through aging. At least three distinct populations could be detected by the surface expression of CD206 and MHC II, where the cells transiently expressed either one or none of them. Also, few cells expressed both CD206 and MHC II. The unsupervised, self-organizing, map algorithm revealed even higher heterogeneity among the testicular macrophages as a subpopulation of the CD206^{+} cells co-expressed CSF1R (CD115). (III: Fig 2a-d,

SFig 3a-b) The in silico lineage tracing analysis revealed clear trajectories between the macrophage subpopulations, but those were not observed between the macrophages and Ly6C^{low} or Ly6C^{high} monocytes whose frequencies were also low in all the studied time points. Moreover, CD206^{+} MHC II$^{–}$ and CD206$^{–}$ MHC II$^{–}$ cells were labelled by Ki67 indicating proliferation among those populations. (III: Fig 2e-f, SFig 3c & SFig 4a-c) These results may indicate local renewal of the macrophage populations instead of replenishment by bone marrow monocyte derived macrophages.

5.3.3 Macrophage subpopulations localize distinctively and have altered functions (III)

We decided to study the three main macrophage populations (CD206^{+} MHC II$^{–}$, CD206$^{–}$ MHC II$^{–}$ and CD206$^{–}$ MHC II^{+}) as they were identifiable in both mass cytometry and in conventional fluorescence-based flow cytometry (III: Fig 3a, SFig 5a-b). Phagocytosis is one of the best-known functions of macrophages, and we tested this on each of the testicular macrophage populations. We preselected CD11b cells, fed them in vitro with fluorescently labelled dextran, and then analysed the uptake by each macrophage population with flow cytometry. All the analysed macrophages performed phagocytosis, but CD206^{+} cells were the most efficient uptakers as shown by the fold change for the geometric mean of intensity of the signal from dextran. The CD206^{+} cells were also superior in eating the different particles when the payloads were administered in vivo to the vasculature. This result is in line with our observation that the CD206^{+} cells were in the vicinity of the CD31^{+} blood vessels while the MHC II^{+} cells were sitting on top of the seminiferous tubules having minimal access to the particles. Although the MHC II expression in testicular macrophages is only detected after two weeks of life, peritubular MHC II$^{–}$ macrophages with elongating dendrite-like processes we already saw in one-week-old mice. (III: Fig 3c-h, SFig 5c-d)

5.3.4 Testis macrophages are largely of foetal monocyte origin (III)

In order to examine the origins of the different macrophage populations found in the testis after birth, we took advantage of the well-described inducible lineage tracing mouse models, *Csf1r*$^{Mer\text{-}iCre\text{-}Mer}$ or *CX3CR1*^{CreERT2} , and crossed them with *R26R-EYFP* reporter mice. Both are known to label yolk sac-derived primitive macrophages and their progeny when induced on specific days (Ginhoux et al. 2010; Goldmann et al. 2016). Flow cytometry analyses showed that testicular CD45^{low} F4/80^{high} cells were labelled before or just after the birth and that the labelled cells

persisted in adult testes, but were highly diluted. This finding was supported by the results from the experiment where we depleted yolk sac-derived macrophages from the embryos by CSF1R antibody treatment at E6.5 (Squarzoni et al. 2014). We observed that CD45^{low} F4/80^{high} macrophages were abolished from the testis before birth, but normal numbers of resident macrophages were present in adult testis. (III: Fig 4a-f, Fig 1d and SFig 6a) Furthermore, we found that PLVAP-deficient pre-pubertal mice had decreased numbers of testicular macrophages, and the frequency of CD206^{+} MHC II$^{–}$ interstitial macrophages was significantly lower compared to the WT control suggesting foetal monocyte origins for these cells (III: Fig 4g, SFig 6b). This hypothesis was supported by the discovery that at E14.5 induced and E16.5 boosted tomato labelling of *Ccr2*$^{+}$ foetal monocytes were detected in 50% of CD206^{+} MHC II$^{–}$ macrophages in two-week-old testis, and in over 50%, 32% and 30% of CD206^{+} MHC II$^{–}$, CD206$^{–}$ MHC II$^{–}$ and CD206$^{–}$ MHC II^{+} macrophages, respectively, in five-week-old testis (**Figure 7**). Taken together, these results show that foetal liver-derived monocytes significantly contribute to the testicular macrophage pool.

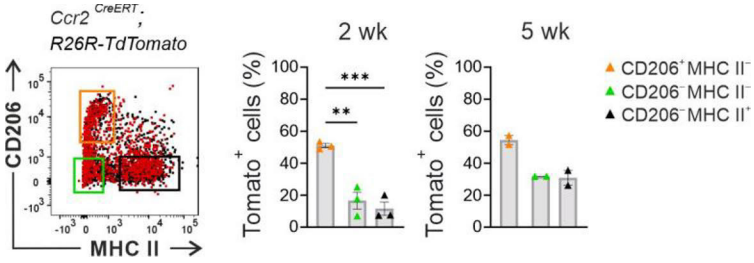

Figure 7. Testicular macrophages are derived from Ccr2+ foetal monocytes (Study III). Representative flow cytometry plot and quantifications of foetal monocyte-derived macrophages in lineage tracing study where Cre-recombinase was induced at E14.5 and boosted at E16.5. Plot shows Tomato+ cells overlaid on top of total acquired cells both gated as viable singlets with CD45+CD11b+F4/80+ and the gating of CD206+ MHC II– (in orange), CD206– MHC II– (in green), and CD206– MHC II+ (in black) macrophages in testis of a five-week-old mouse. In the quantifications each triangle represents one mouse. Data are presented as mean ± SEM and analysed by one-way ANOVA with Tukey's post-hoc test, where **p < 0.01 and ***p < 0.001.

5.3.5 Adult bone marrow-derived macrophages are absent in testis (III)

CD206$^{–}$ MHC II^{+} peritubular macrophages have been suggested to derive from bone marrow monocytes (Mossadegh-Keller et al. 2017), thus we decided to test the hypothesis with well-established *Ccr2*$^{–/–}$ and *Nur*$^{–/–}$ mouse models that manifest with

lack of Ly6C^{high} and Ly6C^{-} monocytes, respectively (Boring et al. 1997; Serbina and Pamer 2006; Hanna et al. 2011). Surprisingly, we observed no decrease in frequencies or cell counts in any of the testicular macrophage populations in either of the models compared to their background-matched controls (III: Fig 4h-I, SFig 6c-d). As these results hinted that bone marrow-derived monocytes would not significantly contribute to a normal testicular macrophage niche, we investigated whether the monocytes even had potential to replenish an emptied niche. To transiently abolish the macrophages and monocytes, we treated WT mice with CSF1 antibody and clodronate containing liposomes. After 11 days of recovery, blood monocytes were circulating in regular numbers and resident kidney macrophages were partially replenished by those monocytes. In contrast, testicular macrophages remained absent. We noticed that CSF1 antibody alone was sufficient to abolish the testicular macrophages, therefore we single treated WT newborn mice with that antibody and analysed the testes macrophage content by flow cytometry after recovery periods of alternating lengths. We found that after extensive 12-week recovery time, interstitial and peritubular macrophage numbers were recovered only partially. Notably, the recovery seems to start in interstitial CD206^{+} population as shown in a five-week-old testis, which is in line with the scheme where the CD206^{+} macrophages would differentiate into the MHC II^{+} peritubular cells as proposed by in silico lineage tracing. (III: Fig 5, SFig 7, SFig 8) Taken together, these results indicate that circulating monocytes contribute minimally to the testis macrophage populations and are dispensable in the replenishment of the emptied testicular macrophage niche.

5.3.6 Macrophages are needed in embryonic era for intact spermatogenesis at puberty (III)

To assess the impact of macrophage depletion on spermatogenesis, we examined the overall morphology of testes and relative numbers of Sertoli cells and Leydig cells in the seminiferous tubules of CSF1 antibody-treated WT and *Ccr2*$^{-/-}$ mice. With these parameters we did not detect any difference between the treated and control mice (III: Fig 6). These results indicate that macrophages or monocytes are not needed for intact spermatogenesis after birth. The question remained if the macrophages are completely dispensable for the normal production of sperm cells. To address this, we did the CSF1R antibody depletion for yolk sac macrophages at E6.5, treated the newborns with CSF1 antibody, and repeated the CSF1 antibody treatment at the age of two weeks to keep the testicular niche empty. After three weeks of recovery, we found that the testes of treated mice were indeed devoid of macrophages and that spermatogenesis was severely impaired. Notably, the postmeiotic sperm cells were significantly lacking and the BTB appeared disrupted.

These results were comparable to those from osteopetrotic *Csf1*^{op} suffering lifelong lack of macrophages. (III: Fig 7) Taken together, these results indicate that foetal macrophages are a necessity for spermatogenesis later in life.

6 Discussion

6.1 Impact of PLVAP deficiency on resident macrophage populations

PLVAP is historically known to regulate the size-selective passage of molecules between blood and tissue stroma. In the previous work of Rantakari et al. (2015), the authors showed that PLVAP forms the diaphragms on TECs found in the lymphatic endothelial cells on the floor of the sinuses in the lymph node. Moreover, they found that lymphocyte migration from the skin via the lymphatics to the draining lymph node was defective in PLVAP-deficient mice, which is in line with a previous study suggesting a role for PLVAP in leukocyte migration through blood vessels (Rantakari et al. 2015; Keuschnigg et al. 2009).

A significant proportion of resident macrophages is derived from foetal precursors in multiple tissues. The primitive macrophages from the yolk sac and monocytic precursors from the foetal liver are both seeding tissues before birth (Hoeffel et al. 2015). The results from **Study I** suggest that PLVAP is needed for the emigration of foetal monocytes from the liver during foetal development, and thus, for generating resident macrophage populations. In our studies, alveolar macrophages in the lungs and Kupffer cells in the liver, both of which others have shown to be derived from foetal monocytes, were lacking in adult *Plvap*$^{-/-}$ mice (Guilliams et al. 2013; Hoeffel et al. 2015). Later, a similar lack of foetal liver-derived macrophages in *Plvap*$^{-/-}$ mice as presented in **Study I** was shown for mammary glands, ovaries, and white adipose tissue (Jäppinen et al. 2019; Jokela et al. 2020; Félix et al. 2021).

Elgueta et al. (2016) reported that endothelial cell-specific depletion of PLVAP leads to a decrease in B1 B cell numbers in the peritoneal cavity and marginal zone (MZ) B cells in the spleen of adult mice. Although they could not determine a specific mechanism, they discussed multiple scenarios of how PLVAP deficiency could cause such an impact. One of them was that the changes in the tissue environment due to leaky vessels of the *Plvap*$^{-/-}$ mice could affect the viability of the leukocytes and their progenitors. Another scenario discussed was that B cell specific surface proteins might interact with PLVAP to enable extravasation to target tissues. However, no evidence has been provided to support those theories.

Intriguingly, the B1 B cells, including MZ B cells that are self-renewing, much like resident macrophages, are derived from the foetal period and seeded to the tissues from the foetal liver (Montecino-Rodriguez, Leathers, and Dorshkind 2006; Yoshimoto et al. 2011). Hence, it would be interesting to study if PLVAP also regulates the seeding of these foetal lymphocytes and, if so, to reveal a more general role for PLVAP in leukocyte transmigration in the foetal liver.

Considering the discovery of the diminished populations of both resident macrophages and B1 B cells in peripheral tissues under PLVAP deficiency, it is puzzling that these populations are widely recognized as self-renewing and are still partly lacking. One might expect that cells with appreciated proliferation capacity would be able to fill the niche over time or that bone marrow monocyte-derived macrophages could fill the niche. For example, Kupffer cells are known to be reconstituted by the monocytes in two weeks after complete depletion in adult mice. Moreover, if the depletion is only partial, leftover Kupffer cells proliferate to compete with monocytes to fill the niche. (Bonnardel et al. 2019; Scott et al. 2016) While the question remains to be thoroughly addressed, a few explanations may be considered. First, the niche establishment may require the pioneering macrophages entering the tissue in the foetal era. The second explanation could be in the altered blood composition of *Plvap*$^{-/-}$ mice as they suffer from severe hypoproteinaemia (Herrnberger, Ebner, et al. 2012; Herrnberger, Seitz, et al. 2012; Stan et al. 2012). The availability of circulating CSF1 (or other chemoattractants), which is needed for the survival and maintenance of resident macrophage populations, may be altered and therefore affect the tissue niche. The third possibility is that the lack of PLVAP in the target tissues changes the endothelium so that leukocytes struggle to enter the tissue.

6.2 Functions of PLVAP beyond sieving

As discussed above, PLVAP is not only limiting the trafficking of macromolecules but is also related to the transcellular migration of the leukocytes. The obvious question is how PLVAP mediates this action. As a heparin-binding protein, PLVAP could generate a concentration of growth factors or chemokines to guide leukocytes that express suitable receptors (Grünewald et al. 2010; Hnasko, McFarland, and Ben-Jonathan 2002). Hence, the leukocytes that are to transmigrate through endothelium could follow this chemotactic gradient to the site of passing. The PLVAP expressing fenestrae/TEC/caveolae are commonly found in the periphery of EC, where the width from luminal to the basal surface is thinnest and where the transcellular migration occurs (Michel and Neal 1999; Wisse 1970; Carman 2009). Leukocytes have been shown to palpate the endothelial surface with invadosome-like protrusions to detect sites for passing (Carman et al. 2007). Keuschnigg et al. (2009)

demonstrated in vitro that neither rolling nor adhesion, but rather the transmigration of leukocytes through TECs surrounded by PLVAP was remarkably abrogated by an anti-PLVAP antibody blockade. Notably, such a defect was not seen in the migration of polymorphonuclear cells known to prefer paracellular migration (Keuschnigg et al. 2009). In **Study I**, we demonstrated that heparin beads bound PLVAP, VEGF, and neuropilin-1 from E14.5 lysed liver. Moreover, our in vitro experiments indicate that PLVAP is associated with VEGF via heparin scaffold and further with neuropilin-1, which was shown to be expressed by the foetal monocytes. Taken together, these observations warrant the speculation that PLVAP might indeed provide a platform for intensive chemotactic signalling that is then detected by the leukocytes as they fumble for the transendothelial pathway. The schematic of this phenomenon is represented in **Figure 8**. Since our hypothesis remains to be formally proven, other explanations can be considered. One would be that the lack of PLVAP and open fenestrae in the foetal liver endothelium causes increased flow of macromolecules, and, due to the increased osmotic pressure, fluids to the tissue. This in turn would make leukocyte migration against the flow to the circulation physically harder.

In the adult LSEC, the PLVAP does not form the diaphragms, as fenestrae appear empty (Bankston and Pino 1980). However, our results from **Study II** demonstrate robust expression of PLVAP in the sinusoidal area of the liver acinus, thereby confirming the earlier observations of *Plvap* expression also in the adult liver. PLVAP is thought to be expressed exclusively in the diaphragms in endothelial cells (Tkachenko et al. 2012). The in vivo labelling of PLVAP via the vascular administration of the anti-PLVAP antibody suggests that the protein is found in the luminal side of the liver vessels. However, the possible endocytosis of the antibody and thereby the intracellular labelling cannot be entirely excluded. Notably, the open fenestrae and the increased leakiness of the vessels in *Plvap*$^{-/-}$ mice encourage the evaluation of whether the antibody flows passively through the fenestrae and labels the adjacent cells, which would be the hepatic stellate cells that have been shown to express PLVAP (Terkelsen et al. 2020).

Interestingly, a recent study demonstrated distinct and specific PLVAP expression in self-renewal biased SSCs in seminiferous tubules of mouse testes (Nakagawa et al. 2021). Even though evidence for the functional significance was lacking, the nonendothelial localization of this protein is a remarkable discovery and hints at a more versatile role for PLVAP in biology overall. Generally, PLVAP likely possesses functions beyond molecule sieving in different tissues, and a broader examination of them is warranted.

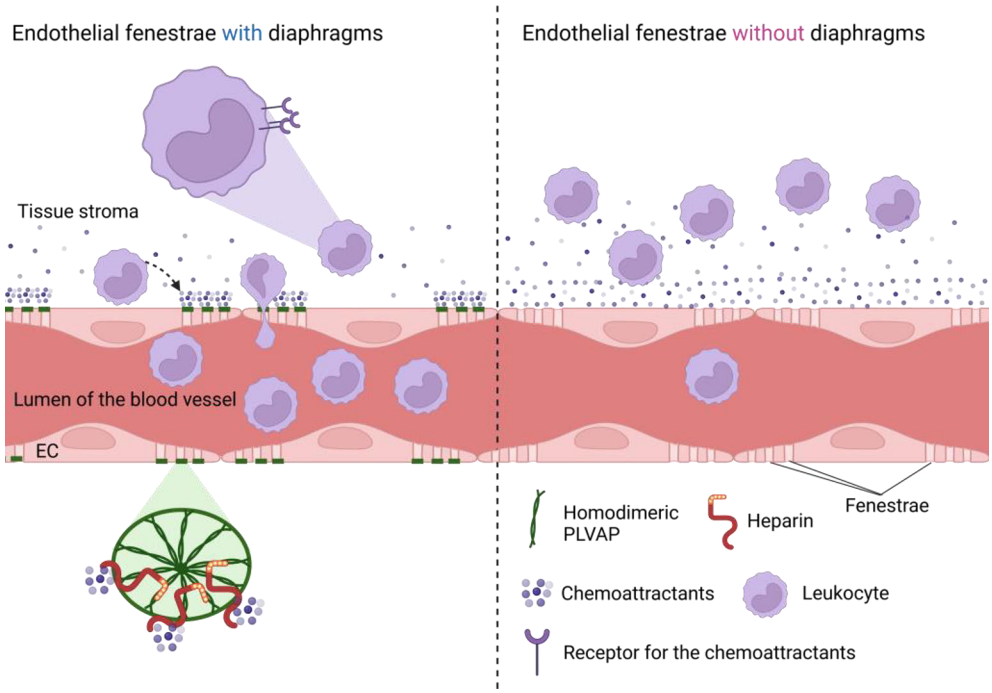

Figure 8. Illustration of the hypothetical role of PLVAP in the transendothelial exit of leukocytes from the foetal liver to blood circulation. In diaphragmal fenestrae, the chemoattractants can bind PLVAP via a heparin bridge and create a concentrated signal for the adhered leukocytes searching for a pathway to cross the endothelium. Leukocytes expressing receptors for the chemoattractants detect these signals and thus the underlying fenestrae. In the absence of PLVAP and diaphragms the chemoattractants are dispersed in the larger area and therefore fail to guide the leukocytes to the fenestrae. Hence, the transmigration is more efficient in presence than in absence of PLVAP. Endothelial cell, EC. Created with BioRender.com.

6.3 Fenestrae are generated in the absence of PLVAP

Herrnberger et al. (2014) have suggested that PLVAP is needed for the biogenesis of the fenestrae. However, our electron microscopy data from **Studies I** and **II** demonstrate that fenestrae are found in the LSEC of foetal and adult *Plvap*$^{-/-}$ mice. In support of our data, Stan et al. (2012) presented electron micrographs that show empty fenestrae and TECs in endothelial cells under the PLVAP deficiency. Since the fenestrae are found in the *Plvap*$^{-/-}$ mice, it would be interesting to study if the VEGFR-2 and neuropilin-1 associations, shown in **Study II** in the foetal liver of

wild type mouse, are normally generated in absence of PLVAP and if their localization in the LSEC is changed.

6.4 Ontogeny of testicular macrophages

Ever since the resident macrophages in adult mice were discovered to arise from embryonic sources, the yolk sac, or the foetal liver, their ontogeny has been under active investigation. Testicular macrophages, identified by their expression of F4/80, have been shown to localize to the interstitial space, with close contacts with the Leydig cells and the peritubular department along with the PMCs in the mouse testis (Hume et al. 1984). DeFalco et al. (2014) were the first to show that primitive macrophages from the yolk sac are present in the gonadal primordium before the onset of testicular morphogenesis. They showed that macrophages were needed for the vascular remodelling and tissue pruning in the developing testis. Later, Mossadegh-Keller et al. (2017) demonstrated that yolk sac-derived macrophages persisted in the adult testis, although they were highly diluted by age. Our lineage tracing data from **Study III** support their discoveries that yolk sac-derived macrophages contribute to the macrophage pool in the testis anlage and persist in the niche until adulthood in decreasing proportions.

In the aforementioned studies by others, the foetal monocyte contribution to the testis macrophage population remains an open question (DeFalco et al. 2014; Mossadegh-Keller et al. 2017). Our results from five-week-old *Plvap*$^{-/-}$ mice suggest a remarkable contribution from the foetal monocytes to the testicular macrophage pool, as a significant decrease in CD206^{+} MHC II^{-} and CD206^{-} MHC II^{-} cell numbers was observed in the knockout mice. In addition, the preliminary supplemental data on *Ccr2* reporter mice suggest that monocytic precursors from the foetal liver constitute a substantial proportion of, if not the entire, testis macrophage population. Although, to conclude this hypothesis, further investigation of the kinetics of these labelled cells at earlier and later time points are required. The recent publications by DeFalco and colleagues (Gu et al. 2023) and Wang et al. (2021) confirmed, based on their lineage tracing experiments on the *Flt3*^{Cre} and c-Kit^{CreER} reporter mice, that the descendants from the foetal liver haematopoiesis are the main constituents of the testicular macrophage pool.

Bone marrow monocyte infiltration to the testis in juvenile mice has been proposed to solely constitute the MHC II^{+} peritubular macrophages (Mossadegh-Keller et al. 2017). This conclusion was drawn based on the adoptive transfusion of bone marrow from the universal reporter mouse to the liver of newborn C57BL/6 mice, which resulted in an observation of increasing proportions of labelled macrophages, especially in those expressing MHC II. According to their proposed working model, the donor's bone marrow-derived haematopoietic stem and

progenitor cells would colonize the recipient's bone marrow and produce labelled monocytic progeny. Indeed, successful colonization of the bone marrow was demonstrated. However, since total bone marrow transfer was used instead of purified HSPCs, the possible donor-derived monocyte infiltration to neonatal testis cannot be excluded. Notably, adult bone marrow-derived monocytes have been shown to compete with the foetal monocytes and partially colonize the alveolar macrophage niche in the lung and the Kupffer cell niche in the liver in a similar type of experimentation (van de Laar et al. 2016; Scott et al. 2016).

Our data from CCR2 and Nur77 deficient mice with decreased numbers of classical Ly6C^{+} (Boring et al. 1997) and patrolling Ly6C$^{low/-}$ (Hanna et al. 2011) monocytes, respectively, showed normal or even increased numbers of peritubular MHC II^{+} macrophages. Moreover, after thorough depletion of macrophages from the testis, the slow recovery hints that circulating monocytes are not the primary source for testicular macrophages. Instead, an accelerated local proliferation of depletion-resistant resident macrophages seems to cover the replenishment of the niche. Supporting evidence for the minimal contribution from adult bone marrow-derived monocytes to testicular macrophage pool in steady state was demonstrated in multiple ways, including long-term parabiosis, bone marrow chimeras, *Ccr2*$^{-/-}$ mice, and *Ms4a3*^{Cre} reporter mice by Wang et al. (2021). They also showed that circulating monocytes were able to enter the testis macrophage niche depleted by irradiation, although only <10% of the recovered macrophages were donor derived after five weeks of recovery and only a slight increase to that was observed after an extensive 12 weeks of recovery. Irradiation has been shown to induce endothelial permeability and an increased expression of chemotactic signals, such as CCL2, even in the brain, allowing circulating monocyte infiltration (Mildner et al. 2007). A similar phenomenon may occur in the testis as well and should be noted. Notably, testicular microvasculature has been reported to have a nonfenestrated continuous endothelium lining lacking PLVAP expression but showing an increased expression for the BBB associated markers in rats (Holash et al. 1993; Stan et al. 1999). In support of that, the transcriptome profile of the testicular endothelium in mice was recently discovered to resemble that of brain tissue more than the other tissues studied (Kalucka et al. 2020). Thus, the testis may be harder to infiltrate by the circulating leukocytes. Together these observations support the hypothesis that testis is a tightly restricted site with minimal circulating monocyte infiltration in a steady state.

6.5 Significance of the resident macrophages for testis function

Resident macrophages in the testes have been proposed to participate in the morphogenesis of the tissue, the regulation of the immune environment, and in steroidogenesis and even spermatogenesis (Mossadegh-Keller and Sieweke 2018). Yolk sac-derived primitive macrophages are needed for correct vascularization in E13.5 testes as shown in *Cx3cr1-Cre; Rosa-DTA* model (DeFalco et al. 2014). We depleted those cells successfully with a CSF1R antibody administration at E6.5 and observed unaltered numbers of the foetal monocyte-derived macrophages compared to control mice in **Study III**. In five-week-old mice we observed unchanged numbers of testicular macrophages. The same phenomenon has been demonstrated to occur in several other tissues as well (Hoeffel et al. 2015). In the recent publication from DeFalco and associates (Gu et al. 2023), the authors replicated the CSF1R antibody depletion experiment and examined the effects on the testicular cord formation. They saw defects in the counts of testis cords at E18.5, which concurs with their previous data showing that yolk sac-derived macrophages are involved in the cord formation process (DeFalco et al. 2014). The question remains if this has an impact on the testis function later in life.

Others have implicated that the testis macrophages are needed for intact spermatogenesis, either through a contribution to the testosterone production by Leydig cells or to the retinoic acid synthesis (DeFalco et al. 2015). The osteopetrotic CSF1-deficient mouse model, manifesting with minimal or nonexistent macrophage counts in several tissues, including the testis, has been shown to suffer from low levels of intratesticular testosterone, infertility, and low libido (Cohen, Hardy, and Pollard 1997; Cohen et al. 1996). DeFalco et al. (2015) executed systemic depletion of macrophages in *Cx3Cr1*-Cre; *Rosa*-iDTR mice and observed an interrupted differentiation of spermatogonia but only a 50% decrease in the testicular testosterone levels. Notably, they achieved a 90% depletion efficiency and rapid, complete recovery of the populations by 14 days in three-month-old mice. Surprisingly, our data from **Study III** hint that the macrophages depleted after birth are not needed for complete spermatogenesis. After five weeks of recovery, only the interstitial $CD206^{+}$ testicular macrophages had started to partially recover. Our findings are subject to the interpretation that the recovered cells are sufficient to fulfill the tasks of macrophages related to spermatogenesis. However, long term treatment (six weeks) with CSF1R antibody in adult mice by Sauter et al. (2014) was shown to deplete the testicular macrophages without disturbing spermatogenesis. In addition, normal levels of testosterone were detected in the serum (Sauter et al. 2014). The different methodology and their systemic impact may partially explain the varying observations. Notably, low testosterone levels correlate with sub- or infertility (McLachlan et al. 2002). Therefore, testosterone and LH levels should be

thoroughly tested, as the systemic depletion of macrophages in our and other's studies may have affected the hypothalamic–pituitary–gonadal axis. On the other hand, the sudden death of almost all cells from the second-largest population of testicular interstitium in DeFalco's study (2015) likely has triggered an inflammatory response which is known to affect spermatogenesis (Zhao et al. 2014).

Although spermatogenesis seemed normal in the mice with transient depletion of macrophages perinatally, very different results were obtained when we combined yolk sac-derived macrophage depletion with CSF1R antibody and postnatal macrophage depletion with CSF1 antibody. Spermatogenesis was impaired and only a few elongating spermatids were observed from the histological cross-sections. Also, the BTB seemed compromised. However, Leydig cell and Sertoli cell numbers were comparable to the controls. These results imply that macrophages in the foetal era are crucial for intact spermatogenesis. While the exact developmental impact of macrophages needed for the normal testis function remains to be discovered, some possible conclusions can be discussed. One possibility is that the developmental defects in the testicular cord formation described in the E18.5 testis in the absence of yolk sac-derived macrophages by Gu et al. (2023) combined with a prolonged lack of macrophages in the niche, are leading to the defects in adult mice we observed in **Study III**. Another possibility is that the antibody treatments affected the SSCs directly, as they express CSF1R too. Although, according to in vitro data, the lack of CSF1 would affect the proliferation of SSC, not much is known about the effects in vivo. (Oatley et al. 2009; Kokkinaki et al. 2009) Finally, the possible interference with the hypothalamic–pituitary–gonadal axis may lead to hormonal changes that cause the observed effects.

In **Study III**, we concentrated on examining the testicular macrophage populations in a steady state, but their role in inflammation would be interesting to study in the future. As mentioned earlier, testis macrophages are suggested to maintain the immune privilege in the testis (Fijak, Bhushan, and Meinhardt 2016). Indeed, both interstitial and peritubular macrophages have transcriptional profiles for immune suppression as shown by others (Mossadegh-Keller et al. 2017). In addition, they possibly contribute to testosterone production by providing source material for Leydig cells (Nes et al. 2000). Testosterone is known to promote immune suppression in testis (Fijak, Bhushan, and Meinhardt 2016). Moreover, MHC II expressed by the peritubular macrophages hints at an antigen presentation potential, which may be related to immune tolerance and/or to the promotion of inflammation in case of an intratesticular pathogen attack.

7 Conclusions

Here we present a new molecule participating in the transendothelial migration of the monocytes in the foetal liver endothelial cells. Furthermore, we show that the molecular dynamics in the liver sinusoidal endothelium are changed after birth. Finally, we describe the ontogeny and functional significance of testicular resident macrophages. These results provide insight into resident macrophage kinetics in general, and in the testis in particular. Our data also reveals unexpected localization for the well-established blood endothelial protein. The summary of the key discoveries from the studies are the following:

1. PLVAP regulates the transendothelial migration of the foetal monocytes in liver.
2. PLVAP deficiency results in reduced numbers of the foetal liver-derived macrophages in the tissues of adult mice.
3. PLVAP associates with chemotactic agents in the foetal liver endothelium.
4. PLVAP persists in the absence of diaphragms in the adult LSEC in mice.
5. The molecular interaction partners of PLVAP in the LSEC are different before and after birth.
6. Testicular-resident macrophages are derived largely from the monocytes from the foetal liver.
7. Resident macrophages in the foetal era are needed for intact spermatogenesis upon puberty.

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References

Agostino, P., S. Milano, C. Barbera, G. Di Bella, M. La Rosa, V. Ferlazzo, R. Farruggio, et al. 1999. "Sex Hormones Modulate Inflammatory Mediators Produced by Macrophages." *Annals of the New York Academy of Sciences* 876 (1): 426–29. https://doi.org/10.1111/j.1749-6632.1999.tb07667.x.

Aird, William C. 2007. "Phenotypic Heterogeneity of the Endothelium." *Circulation Research* 100 (2): 158–73. https://doi.org/10.1161/01.RES.0000255691.76142.4A.

Ajami, Bahareh, Jami L. Bennett, Charles Krieger, Wolfram Tetzlaff, and Fabio M.V. Rossi. 2007. "Local Self-Renewal Can Sustain CNS Microglia Maintenance and Function throughout Adult Life." *Nature Neuroscience 2007 10:12* 10 (12): 1538–43. https://doi.org/10.1038/nn2014.

Alam, Muhammad S. 2022. "Proximity Ligation Assay (PLA)." *Methods in Molecular Biology* 2422: 191–201. https://doi.org/10.1007/978-1-0716-1948-3_13/FIGURES/2.

Anton, F., C. Morales, R. Aguilar, C. Bellido, E. Aguilar, and F. Gayín. 1998. "A Comparative Study of Mast Cells and Eosinophil Leukocytes in the Mammalian Testis." *Journal of Veterinary Medicine Series A* 45 (1–10): 209–18. https://doi.org/10.1111/J.1439-0442.1998.TB00819.X.

Auffray, Cedric, Darin Fogg, Meriem Garfa, Gaelle Elain, Olivier Join-Lambert, Samer Kayal, Sabine Sarnacki, Ana Cumano, Gregoire Lauvau, and Frederic Geissmann. 2007. "Monitoring of Blood Vessels and Tissues by a Population of Monocytes with Patrolling Behavior." *Science* 317 (5838): 666–70. https://doi.org/10.1126/science.1142883.

Austyn, Jonathan M., and Siamon Gordon. 1981. "F4/80, a Monoclonal Antibody Directed Specifically against the Mouse Macrophage." *European Journal of Immunology* 11 (10): 805–15. https://doi.org/10.1002/EJI.1830111013.

Bain, Calum C., Alberto Bravo-Blas, Charlotte L. Scott, Elisa Gomez Perdiguero, Frederic Geissmann, Sandrine Henri, Bernard Malissen, Lisa C. Osborne, David Artis, and Allan Mc I. Mowat. 2014. "Constant Replenishment from Circulating Monocytes Maintains the Macrophage Pool in the Intestine of Adult Mice." *Nature Immunology 2014 15:10* 15 (10): 929–37. https://doi.org/10.1038/ni.2967.

Bain, Calum C., Catherine A. Hawley, Hannah Garner, Charlotte L. Scott, Anika Schridde, Nicholas J. Steers, Matthias Mack, et al. 2016. "Long-Lived Self-Renewing Bone Marrow-Derived Macrophages Displace Embryo-Derived Cells to Inhabit Adult Serous Cavities." *Nature Communications 2016 7:1* 7 (1): 1–14. https://doi.org/10.1038/ncomms11852.

Bankston, Patrick W., and Richard M. Pino. 1980. "The Development of the Sinusoids of Fetal Rat Liver: Morphology of Endothelial Cells, Kupffer Cells, and the Transmural Migration of Blood Cells into the Sinusoids." *American Journal of Anatomy* 159 (1): 1–15. https://doi.org/10.1002/AJA.1001590102.

Basset, Christelle, John Holton, Rachel O'Mahony, and Ivan Roitt. 2003. "Innate Immunity and Pathogen–Host Interaction." *Vaccine* 21 (SUPPL. 2): S12–23. https://doi.org/10.1016/S0264-

410X(03)00195-6.

Bearer, Elaine L., and Lelio Orci. 1985. “Endothelial Fenestral Diaphragms: A Quick-Freeze, Deep-Etch Study.” *Journal of Cell Biology* 100 (2): 418–28. https://doi.org/10.1083/JCB.100.2.418.

Benz, Claudia, Vera C. Martins, Freddy Radtke, and Conrad C. Bleul. 2008. “The Stream of Precursors That Colonizes the Thymus Proceeds Selectively through the Early T Lineage Precursor Stage of T Cell Development.” *Journal of Experimental Medicine* 205 (5): 1187–99. https://doi.org/10.1084/JEM.20072168.

Benz, Fabienne, Viraya Wichitnaowarat, Martin Lehmann, Raoul Fv Germano, Diana Mihova, Jadranka Macas, Ralf H. Adams, et al. 2019. “Low Wnt/β-Catenin Signaling Determines Leaky Vessels in the Subfornical Organ and Affects Water Homeostasis in Mice.” *ELife* 8 (April). https://doi.org/10.7554/ELIFE.43818.

Bhang, Dong Ha, Bang Jin Kim, Byung Gak Kim, Keri Schadler, Kwan Hyuck Baek, Yong Hee Kim, Wayland Hsiao, et al. 2018. “Testicular Endothelial Cells Are a Critical Population in the Germline Stem Cell Niche.” *Nature Communications 2018 9:1* 9 (1): 1–16. https://doi.org/10.1038/s41467-018-06881-z.

Bhushan, Sudhanshu, María S. Theas, Vanesa A. Guazzone, Patricia Jacobo, Ming Wang, Monika Fijak, Andreas Meinhardt, and Livia Lustig. 2020. “Immune Cell Subtypes and Their Function in the Testis.” *Frontiers in Immunology* 11 (September). https://doi.org/10.3389/FIMMU.2020.583304.

Bian, Zhilei, Yandong Gong, Tao Huang, Christopher Z.W. Lee, Lihong Bian, Zhijie Bai, Hui Shi, et al. 2020. “Deciphering Human Macrophage Development at Single-Cell Resolution.” *Nature 2020 582:7813* 582 (7813): 571–76. https://doi.org/10.1038/s41586-020-2316-7.

Biben, C., T. S. Weber, K. S. Potts, J. Choi, D. C. Miles, A. Carmagnac, T. Sargeant, et al. 2023. “In Vivo Clonal Tracking Reveals Evidence of Haemangioblast and Haematomesoblast Contribution to Yolk Sac Haematopoiesis.” *Nature Communications 2023 14:1* 14 (1): 1–14. https://doi.org/10.1038/s41467-022-35744-x.

Blériot, Camille, Svetoslav Chakarov, and Florent Ginhoux. 2020. “Determinants of Resident Tissue Macrophage Identity and Function.” *Immunity* 52 (6): 957–70. https://doi.org/10.1016/J.IMMUNI.2020.05.014.

Blevins, G., and S. Fedoroff. 1995. “Microglia in Colony-stimulating Factor 1-deficient Op/Op Mice.” *Journal of Neuroscience Research* 40 (4): 535–44. https://doi.org/10.1002/JNR.490400412.

Böiers, Charlotta, Joana Carrelha, Michael Lutteropp, Sidinh Luc, Joanna C.A. Green, Emanuele Azzoni, Petter S. Woll, et al. 2013. “Lymphomyeloid Contribution of an Immune-Restricted Progenitor Emerging Prior to Definitive Hematopoietic Stem Cells.” *Cell Stem Cell* 13 (5): 535–48. https://doi.org/10.1016/J.STEM.2013.08.012.

Bonnardel, Johnny, Wouter T’Jonck, Djoere Gaublomme, Robin Browaeys, Charlotte L. Scott, Liesbet Martens, Bavo Vanneste, et al. 2019. “Stellate Cells, Hepatocytes, and Endothelial Cells Imprint the Kupffer Cell Identity on Monocytes Colonizing the Liver Macrophage Niche.” *Immunity* 51 (4): 638-654.e9. https://doi.org/10.1016/J.IMMUNI.2019.08.017.

Boring, Landin, Jennifa Gosling, Stephen W. Chensue, Steven L. Kunkel, Robert V. Farese, Hal E. Broxmeyer, and Israel F. Charo. 1997. “Impaired Monocyte Migration and Reduced Type 1 (Th1) Cytokine Responses in C-C Chemokine Receptor 2 Knockout Mice.” *Journal of Clinical Investigation* 100 (10): 2552–61. https://doi.org/10.1172/JCI119798.

Braet, Filip, and Eddie Wisse. 2002. “Structural and Functional Aspects of Liver Sinusoidal Endothelial Cell Fenestrae: A Review.” *Comparative Hepatology* 1 (1): 1–17. https://doi.org/10.1186/1476-5926-1-1/FIGURES/6.

Broekaert, Ilse Julia, Kerstin Becker, Ingo Gottschalk, Friederike Körber, Jörg Dötsch, Holger Thiele,

Janine Altmüller, Peter Nürnberg, Christoph Hünseler, and Sebahattin Cirak. 2018. “Mutations in Plasmalemma Vesicle-Associated Protein Cause Severe Syndromic Protein-Losing Enteropathy.” *Journal of Medical Genetics* 55 (9): 637–40. https://doi.org/10.1136/JMEDGENET-2018-105262.

Buch, Thorsten, Frank L. Heppner, Christine Tertilt, Tobias J.A.J. Heinen, Marcel Kremer, F. Thomas Wunderlich, Steffen Jung, and Ari Waisman. 2005. “A Cre-Inducible Diphtheria Toxin Receptor Mediates Cell Lineage Ablation after Toxin Administration.” *Nature Methods 2005 2:6* 2 (6): 419–26. https://doi.org/10.1038/nmeth762.

Burn, Garth Lawrence, Alessandro Foti, Gerben Marsman, Dhiren Ferise Patel, and Arturo Zychlinsky. 2021. “The Neutrophil.” *Immunity* 54 (7): 1377–91. https://doi.org/10.1016/J.IMMUNI.2021.06.006.

Busch, Katrin, Kay Klapproth, Melania Barile, Michael Flossdorf, Tim Holland-Letz, Susan M. Schlenner, Michael Reth, Thomas Höfer, and Hans Reimer Rodewald. 2015. “Fundamental Properties of Unperturbed Haematopoiesis from Stem Cells in Vivo.” *Nature 2015 518:7540* 518 (7540): 542–46. https://doi.org/10.1038/nature14242.

Carlin, Leo M., Efstathios G. Stamatiades, Cedric Auffray, Richard N. Hanna, Leanne Glover, Gema Vizcay-Barrena, Catherine C. Hedrick, H. Terence Cook, Sandra Diebold, and Frederic Geissmann. 2013. “Nr4a1-Dependent Ly6Clow Monocytes Monitor Endothelial Cells and Orchestrate Their Disposal.” *Cell* 153 (2): 362–75. https://doi.org/10.1016/J.CELL.2013.03.010.

Carman, Christopher V. 2009. “Mechanisms for Transcellular Diapedesis: Probing and Pathfinding by `invadosome-like Protrusions’.” *Journal of Cell Science* 122 (17): 3025–35. https://doi.org/10.1242/JCS.047522.

Carman, Christopher V., Peter T. Sage, Tracey E. Sciuto, Miguel A. de la Fuente, Raif S. Geha, Hans D D. Ochs, Harold F. Dvorak, Ann M. Dvorak, and Timothy A. Springer. 2007. “Transcellular Diapedesis Is Initiated by Invasive Podosomes.” *Immunity* 26 (6): 784. https://doi.org/10.1016/J.IMMUNI.2007.04.015.

Carreira, Carla Mouta, Selim M Nasser, Di Tomaso, Timothy P Padera, Yves Boucher, Stanislav I Tomarev, and Rakesh K Jain. 2001. “LYVE-1 Is Not Restricted to the Lymph Vessels: Expression in Normal Liver Blood Sinusoids and Down-Regulation in Human Liver Cancer and Cirrhosis 1.” *Cancer Research* 61 (22): 8079–84. http://aacrjournals.org/cancerres/article-pdf/61/22/8079/2488936/ch2201008079.pdf.

Carrero, Javier A., Derrick P. McCarthy, Stephen T. Ferris, Xiaoxiao Wan, Hao Hu, Bernd H. Zinselmeyer, Anthony N. Vomund, and Emil R. Unanue. 2017. “Resident Macrophages of Pancreatic Islets Have a Seminal Role in the Initiation of Autoimmune Diabetes of NOD Mice.” *Proceedings of the National Academy of Sciences of the United States of America* 114 (48): E10418–27. https://doi.org/10.1073/PNAS.1713543114/SUPPL_FILE/PNAS.201713543SI.PDF.

Carson-Walter, Eleanor B., Jessica Hampton, Eveline Shue, Daniel M. Geynisman, Pramod Kumar Pillai, Ramasri Sathanoori, Stephen L. Madden, Ronald L. Hamilton, and Kevin A. Walter. 2005. “Plasmalemmal Vesicle Associated Protein-1 Is a Novel Marker Implicated in Brain Tumor Angiogenesis.” *Clinical Cancer Research* 11 (21): 7643–50. https://doi.org/10.1158/1078-0432.CCR-05-1099.

Ceccacci, Elena, Emanuela Villa, Fabio Santoro, Saverio Minucci, Christiana Ruhrberg, and Alessandro Fantin. 2023. “A Refined Single Cell Landscape of Haematopoiesis in the Mouse Foetal Liver.” *J. Dev. Biol*. https://doi.org/10.3390/jdb11020015.

Cecchini, Marco G., Melissa G. Dominguez, Simonetta Mocci, Antoinette Wetterwald, Rolf Felix, Herbert Fleisch, Orin Chisholm, Willy Hofstetter, Jeffrey W. Pollard, and E. Richard Stanley.

1994. “Role of Colony Stimulating Factor-1 in the Establishment and Regulation of Tissue Macrophages during Postnatal Development of the Mouse.” *Development* 120 (6): 1357–72. https://doi.org/10.1242/DEV.120.6.1357.

Chang, Tao Hsin, Fu Lien Hsieh, Xiaowu Gu, Philip M. Smallwood, Jennifer M. Kavran, Sandra B. Gabelli, and Jeremy Nathans. 2023. “Structural Insights into Plasmalemma Vesicle-Associated Protein (PLVAP): Implications for Vascular Endothelial Diaphragms and Fenestrae.” *Proceedings of the National Academy of Sciences of the United States of America* 120 (14): e2221103120. https://doi.org/10.1073/PNAS.2221103120/SUPPL_FILE/PNAS.2221103120.SAPP.PDF.

Chen, Haolin, Erin Stanley, Shiying Jin, and Barry R. Zirkin. 2010. “Stem Leydig Cells: From Fetal to Aged Animals.” *Birth Defects Research Part C: Embryo Today: Reviews* 90 (4): 272–83. https://doi.org/10.1002/BDRC.20192.

Chen, Liang Yu, Paula R. Brown, William B. Willis, and Edward M. Eddy. 2014. “Peritubular Myoid Cells Participate in Male Mouse Spermatogonial Stem Cell Maintenance.” *Endocrinology* 155 (12): 4964–74. https://doi.org/10.1210/EN.2014-1406.

Chen, Michael J., Tomomasa Yokomizo, Brandon M. Zeigler, Elaine Dzierzak, and Nancy A. Speck. 2009. “Runx1 Is Required for the Endothelial to Haematopoietic Cell Transition but Not Thereafter.” *Nature 2009 457:7231* 457 (7231): 887–91. https://doi.org/10.1038/nature07619.

Cheng, Jade P.X., and Benjamin J. Nichols. 2016. “Caveolae: One Function or Many?” *Trends in Cell Biology* 26 (3): 177–89. https://doi.org/10.1016/J.TCB.2015.10.010.

Christensen, Julie L., and Irving L. Weissman. 2001. “Flk-2 Is a Marker in Hematopoietic Stem Cell Differentiation: A Simple Method to Isolate Long-Term Stem Cells.” *Proceedings of the National Academy of Sciences of the United States of America* 98 (25): 14541–46. https://doi.org/10.1073/PNAS.261562798/ASSET/93D59A6D-4FB6-4D8A-A3AA-F5712AA9B0C6/ASSETS/GRAPHIC/PQ2615627004.JPEG.

Christensen, Julie L., Douglas E. Wright, Amy J. Wagers, and Irving L. Weissman. 2004. “Circulation and Chemotaxis of Fetal Hematopoietic Stem Cells.” *PLOS Biology* 2 (3): e75. https://doi.org/10.1371/JOURNAL.PBIO.0020075.

Cohen, Paula E., Matthew P. Hardy, and Jeffrey W. Pollard. 1997. “Colony-Stimulating Factor-1 Plays a Major Role in the Development of Reproductive Function in Male Mice.” *Molecular Endocrinology* 11 (11): 1636–50. https://doi.org/10.1210/MEND.11.11.0009.

Cohen, Paula E, Orin Chisholm, Robert J Arceci, E Richard Stanley, and Jeffrey W Pollard. 1996. “Absence of Colony-Stimulating Factor-1 in Osteopetrotic (CsfmoP/CsfmOP) Mice Results in Male Fertility Defects’.” *BIOLOGY OF REPRODUCTION* 55: 310–17. https://academic.oup.com/biolreprod/article/55/2/310/2760479.

Creasy, Dianne M., and Robert E. Chapin. 2013. “Male Reproductive System.” *Haschek and Rousseaux’s Handbook of Toxicologic Pathology, Third Edition: Volume 1-3* 1–3 (January): 2493–2598. https://doi.org/10.1016/B978-0-12-415759-0.00059-5.

Croxford, Andrew L., Margit Lanzinger, Felix J. Hartmann, Bettina Schreiner, Florian Mair, Pawel Pelczar, Björn E. Clausen, Steffen Jung, Melanie Greter, and Burkhard Becher. 2015. “The Cytokine GM-CSF Drives the Inflammatory Signature of CCR2+ Monocytes and Licenses Autoimmunity.” *Immunity* 43 (3): 502–14. https://doi.org/10.1016/J.IMMUNI.2015.08.010.

Dai, Xu Ming, Gregory R. Ryan, Andrew J. Hapel, Melissa G. Dominguez, Robert G. Russell, Sara Kapp, Vonetta Sylvestre, and E. Richard Stanley. 2002. “Targeted Disruption of the Mouse Colony-Stimulating Factor 1 Receptor Gene Results in Osteopetrosis, Mononuclear Phagocyte Deficiency, Increased Primitive Progenitor Cell Frequencies, and Reproductive Defects.” *Blood* 99 (1): 111–20. https://doi.org/10.1182/BLOOD.V99.1.111.

Dai, Xu Ming, Xiao Hua Zong, Vonetta Sylvestre, and E. Richard Stanley. 2004. “Incomplete Restoration of Colony-Stimulating Factor 1 (CSF-1) Function in CSF-1–Deficient Csf1op/Csf1op Mice by Transgenic Expression of Cell Surface CSF-1.” *Blood* 103 (3): 1114–23. https://doi.org/10.1182/BLOOD-2003-08-2739.

Danielian, Paul S., David Muccino, David H. Rowitch, Simon K. Michael, and Andrew P. McMahon. 1998. “Modification of Gene Activity in Mouse Embryos in Utero by a Tamoxifen-Inducible Form of Cre Recombinase.” *Current Biology* 8 (24): 1323-S2. https://doi.org/10.1016/S0960-9822(07)00562-3.

DeFalco, T., I. Bhattacharya, A. V. Williams, D. M. Sams, and B. Capel. 2014. “Yolk-Sac-Derived Macrophages Regulate Fetal Testis Vascularization and Morphogenesis.” *Proceedings of the National Academy of Sciences* 111 (23): E2384–93. https://doi.org/10.1073/pnas.1400057111.

DeFalco, Tony, Sarah J. Potter, Alyna V. Williams, Brittain Waller, Matthew J. Kan, and Blanche Capel. 2015. “Macrophages Contribute to the Spermatogonial Niche in the Adult Testis.” *Cell Reports* 12 (7): 1107–19. https://doi.org/10.1016/j.celrep.2015.07.015.

Dick, Sarah A., Anthony Wong, Homaira Hamidzada, Sara Nejat, Robert Nechanitzky, Shabana Vohra, Brigitte Mueller, et al. 2022. “Three Tissue Resident Macrophage Subsets Coexist across Organs with Conserved Origins and Life Cycles.” *Science Immunology* 7 (67): 7777. https://doi.org/10.1126/SCIIMMUNOL.ABF7777/SUPPL_FILE/SCIIMMUNOL.ABF7777_MDAR_REPRODUCIBILITY_CHECKLIST.ZIP.

Easley-Neal, Courtney, Oded Foreman, Neeraj Sharma, Ali A. Zarrin, and Robby M. Weimer. 2019. “CSF1R Ligands IL-34 and CSF1 Are Differentially Required for Microglia Development and Maintenance in White and Gray Matter Brain Regions.” *Frontiers in Immunology* 10 (September): 2199. https://doi.org/10.3389/FIMMU.2019.02199/BIBTEX.

Elgueta, Raul, Dan Tse, Sophie J. Deharvengt, Marcus R. Luciano, Catherine Carriere, Randolph J. Noelle, and Radu V. Stan. 2016. “Endothelial Plasmalemma Vesicle–Associated Protein Regulates the Homeostasis of Splenic Immature B Cells and B-1 B Cells.” *The Journal of Immunology* 197 (10): 3970–81. https://doi.org/10.4049/JIMMUNOL.1501859.

Elkadri, Abdul, Cornelia Thoeni, Sophie J. Deharvengt, Ryan Murchie, Conghui Guo, James D. Stavropoulos, Christian R. Marshall, et al. 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): 381-394.e7. https://doi.org/10.1016/J.JCMGH.2015.05.001.

Epelman, Slava, Kory J. Lavine, Anna E. Beaudin, Dorothy K. Sojka, Javier A. Carrero, Boris Calderon, Thaddeus Brija, et al. 2014. “Embryonic and Adult-Derived Resident Cardiac Macrophages Are Maintained through Distinct Mechanisms at Steady State and during Inflammation.” *Immunity* 40 (1): 91–104. https://doi.org/10.1016/J.IMMUNI.2013.11.019.

Fajgenbaum, David C., and Carl H. June. 2020. “Cytokine Storm.” *New England Journal of Medicine* 383 (23): 2255–73. https://doi.org/10.1056/NEJMRA2026131/SUPPL_FILE/NEJMRA2026131_DISCLOSURES.PDF.

Félix, Inês, Heli Jokela, Joonas Karhula, Noora Kotaja, Eriika Savontaus, Marko Salmi, and Pia Rantakari. 2021. “Single-Cell Proteomics Reveals the Defined Heterogeneity of Resident Macrophages in White Adipose Tissue.” *Frontiers in Immunology* 12 (July): 719979. https://doi.org/10.3389/FIMMU.2021.719979/BIBTEX.

Fijak, Monika, Sudhanshu Bhushan, and Andreas Meinhardt. 2016. “The Immune Privilege of the Testis.” *Immune Infertility: Impact of Immune Reactions on Human Fertility, Second Edition*, January, 97–107. https://doi.org/10.1007/978-3-319-40788-3_5/FIGURES/1.

França, L. R., R. A. Hess, J. M. Dufour, M. C. Hofmann, and M. D. Griswold. 2016. “The Sertoli Cell: One Hundred Fifty Years of Beauty and Plasticity.” *Andrology* 4 (2): 189–212. https://doi.org/10.1111/ANDR.12165.

Furth, R. van, Z. A. Cohn, J. G. Hirsch, J. H. Humphrey, W. G. Spector, and H. L. Langevoort. 1972. “The Mononuclear Phagocyte System: A New Classification of Macrophages, Monocytes, and Their Precursor Cells.” *Bulletin of the World Health Organization* 46 (6): 845. /pmc/articles/PMC2480884/?report=abstract.

Gastfriend, Benjamin D., Hideaki Nishihara, Scott G. Canfield, Koji L. Foreman, Britta Engelhardt, Sean P. Palecek, and Eric V. Shusta. 2021. “Wnt Signaling Mediates Acquisition of Blood-Brain Barrier Properties in Naive Endothelium Derived from Human Pluripotent Stem Cells.” *ELife* 10 (November). https://doi.org/10.7554/ELIFE.70992.

Gautier, Emmanuel L., Tal Shay, Jennifer Miller, Melanie Greter, Claudia Jakubzick, Stoyan Ivanov, Julie Helft, et al. 2012. “Gene-Expression Profiles and Transcriptional Regulatory Pathways That Underlie the Identity and Diversity of Mouse Tissue Macrophages.” *Nature Immunology 2012 13:11* 13 (11): 1118–28. https://doi.org/10.1038/ni.2419.

Gaytan, F., C. Bellido, E. Aguilar, and N. Van Rooijen. 1994. “Requirement for Testicular Macrophages in Leydig Cell Proliferation and Differentiation during Prepubertal Development in Rats.” *Reproduction* 102 (2): 393–99. https://doi.org/10.1530/JRF.0.1020393.

Geissmann, Frederic, Steffen Jung, and Dan R. Littman. 2003. “Blood Monocytes Consist of Two Principal Subsets with Distinct Migratory Properties.” *Immunity* 19 (1): 71–82. https://doi.org/10.1016/S1074-7613(03)00174-2.

Gekas, Christos, Francoise Dieterlen-Lièvre, Stuart H. Orkin, and Hanna K.A. Mikkola. 2005. “The Placenta Is a Niche for Hematopoietic Stem Cells.” *Developmental Cell* 8 (3): 365–75. https://doi.org/10.1016/J.DEVCEL.2004.12.016.

Géraud, Cyrill, Philipp Sebastian Koch, Johanna Zierow, Kay Klapproth, Katrin Busch, Victor Olsavszky, Thomas Leibing, et al. 2017. “GATA4-Dependent Organ-Specific Endothelial Differentiation Controls Liver Development and Embryonic Hematopoiesis.” *The Journal of Clinical Investigation* 127 (3): 1099–1114. https://doi.org/10.1172/JCI90086.

Ginhoux, Florent, Melanie Greter, Marylene Leboeuf, Sayan Nandi, Peter See, Solen Gokhan, Mark F. Mehler, et al. 2010. “Fate Mapping Analysis Reveals That Adult Microglia Derive from Primitive Macrophages.” *Science (New York, N.Y.)* 330 (6005): 841–45. https://doi.org/10.1126/SCIENCE.1194637.

Godin, Isabelle, Françoise Dieterlen-Lièvre, and Ana Cumano. 1995. “Emergence of Multipotent Hemopoietic Cells in the Yolk Sac and Paraaortic Splanchnopleura in Mouse Embryos, Beginning at 8.5 Days Postcoitus.” *Proceedings of the National Academy of Sciences of the United States of America* 92 (3): 773–77. https://doi.org/10.1073/PNAS.92.3.773.

Goldmann, Tobias, Peter Wieghofer, Marta Joana Costa Jordão, Fabiola Prutek, Nora Hagemeyer, Kathrin Frenzel, Lukas Amann, et al. 2016. “Origin, Fate and Dynamics of Macrophages at Central Nervous System Interfaces.” *Nature Immunology 2016 17:7* 17 (7): 797–805. https://doi.org/10.1038/ni.3423.

Gomez Perdiguero, Elisa, Kay Klapproth, Christian Schulz, Katrin Busch, Emanuele Azzoni, Lucile Crozet, Hannah Garner, et al. 2014. “Tissue-Resident Macrophages Originate from Yolk-Sac-Derived Erythro-Myeloid Progenitors.” *Nature 2014 518:7540* 518 (7540): 547–51. https://doi.org/10.1038/nature13989.

Gorukmez, Orhan, Ozlem Gorukmez, and Kaan Demiroren. 2019. “Novel PLVAP Mutation in Protein Losing Enteropathy.” *Fetal and Pediatric Pathology* 38 (6): 534–37. https://doi.org/10.1080/15513815.2019.1627624.

Gosselin, David, Verena M. Link, Casey E. Romanoski, Gregory J. Fonseca, Dawn Z. Eichenfield, Nathanael J. Spann, Joshua D. Stender, et al. 2014. “Environment Drives Selection and Function of Enhancers Controlling Tissue-Specific Macrophage Identities.” *Cell* 159 (6): 1327–40. https://doi.org/10.1016/J.CELL.2014.11.023.

Goyama, Susumu, Go Yamamoto, Munetake Shimabe, Tomohiko Sato, Motoshi Ichikawa, Seishi Ogawa, Shigeru Chiba, and Mineo Kurokawa. 2008. “Evi-1 Is a Critical Regulator for Hematopoietic Stem Cells and Transformed Leukemic Cells.” *Cell Stem Cell* 3 (2): 207–20. https://doi.org/10.1016/J.STEM.2008.06.002.

Grünewald, Felix S., Andrea E. Prota, Alexandra Giese, and Kurt Ballmer-Hofer. 2010. “Structure–Function Analysis of VEGF Receptor Activation and the Role of Coreceptors in Angiogenic Signaling.” *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics* 1804 (3): 567–80. https://doi.org/10.1016/J.BBAPAP.2009.09.002.

Gu, Xiaowei, Anna Heinrich, Shu-Yun Li, and Tony DeFalco. 2023. “Testicular Macrophages Are Recruited during a Narrow Fetal Time Window and Promote Organ-Specific Developmental Functions.” *Nature Communications 2023 14:1* 14 (1): 1–17. https://doi.org/10.1038/s41467-023-37199-0.

Guilliams, Martin, Ismé De Kleer, Sandrine Henri, Sijranke Post, Leen Vanhoutte, Sofie De Prijck, Kim Deswarte, Bernard Malissen, Hamida Hammad, and Bart N. Lambrecht. 2013. “Alveolar Macrophages Develop from Fetal Monocytes That Differentiate into Long-Lived Cells in the First Week of Life via GM-CSF.” *Journal of Experimental Medicine* 210 (10): 1977–92. https://doi.org/10.1084/JEM.20131199.

Guilliams, Martin, Alexander Mildner, and Simon Yona. 2018. “Developmental and Functional Heterogeneity of Monocytes.” *Immunity* 49 (4): 595–613. https://doi.org/10.1016/J.IMMUNI.2018.10.005.

Guilliams, Martin, Guilhem R. Thierry, Johnny Bonnardel, and Marc Bajenoff. 2020. “Establishment and Maintenance of the Macrophage Niche.” *Immunity* 52 (3): 434–51. https://doi.org/10.1016/J.IMMUNI.2020.02.015.

Haldar, Malay, Masako Kohyama, Alex Yick Lun So, Wumesh Kc, Xiaodi Wu, Carlos G. Briseño, Ansuman T. Satpathy, et al. 2014. “Heme-Mediated SPI-C Induction Promotes Monocyte Differentiation into Iron-Recycling Macrophages.” *Cell* 156 (6): 1223–34. https://doi.org/10.1016/J.CELL.2014.01.069.

Hallmann, R., D. N. Mayer, E. L. Berg, R. Broermann, and E. C. Butcher. 1995. “Novel Mouse Endothelial Cell Surface Marker Is Suppressed during Differentiation of the Blood Brain Barrier.” *Developmental Dynamics* 202 (4): 325–32. https://doi.org/10.1002/AJA.1002020402.

Hamilton, B. Jo Nell, Dan Tse, and Radu V. Stan. 2019. “Phorbol Esters Induce PLVAP Expression via VEGF and Additional Secreted Molecules in MEK1-Dependent and P38, JNK and PI3K/Akt-Independent Manner.” *Journal of Cellular and Molecular Medicine* 23 (2): 920–33. https://doi.org/10.1111/JCMM.13993.

Hanna, Richard N., Leo M. Carlin, Harper G. Hubbeling, Dominika Nackiewicz, Angela M. Green, Jennifer A. Punt, Frederic Geissmann, and Catherine C. Hedrick. 2011. “The Transcription Factor NR4A1 (Nur77) Controls Bone Marrow Differentiation and the Survival of Ly6C− Monocytes.” *Nature Immunology 2011 12:8* 12 (8): 778–85. https://doi.org/10.1038/ni.2063.

Hara, Kenshiro, Toshinori Nakagawa, Hideki Enomoto, Mikiko Suzuki, Masayuki Yamamoto, Benjamin D. Simons, and Shosei Yoshida. 2014. “Mouse Spermatogenic Stem Cells Continually Interconvert between Equipotent Singly Isolated and Syncytial States.” *Cell Stem Cell* 14 (5): 658–72. https://doi.org/10.1016/J.STEM.2014.01.019.

Head, Judith R., William B. Neaves, and Rupert E. Billingham. 1983. “Immune Privilege in the Testis.

I. Basic Parameters of Allograft Survival." *Transplantation* 36 (4): 423–31. https://doi.org/10.1097/00007890-198310000-00014.

Heinrich, A., and T. DeFalco. 2020. "Essential Roles of Interstitial Cells in Testicular Development and Function." *Andrology* 8 (4): 903–14. https://doi.org/10.1111/ANDR.12703.

Herrnberger, Leonie, Kathrin Ebner, Benjamin Junglas, and Ernst R. Tamm. 2012. "The Role of Plasmalemma Vesicle-Associated Protein (PLVAP) in Endothelial Cells of Schlemm's Canal and Ocular Capillaries." *Experimental Eye Research* 105 (December): 27–33. https://doi.org/10.1016/J.EXER.2012.09.011.

Herrnberger, Leonie, Robert Hennig, Werner Kremer, Claus Hellerbrand, Achim Goepferich, Hans Robert Kalbitzer, and Ernst R. Tamm. 2014. "Formation of Fenestrae in Murine Liver Sinusoids Depends on Plasmalemma Vesicle-Associated Protein and Is Required for Lipoprotein Passage." *PLOS ONE* 9 (12): e115005. https://doi.org/10.1371/JOURNAL.PONE.0115005.

Herrnberger, Leonie, Roswitha Seitz, Sabrina Kuespert, Michael R. Bösl, Rudolf Fuchshofer, and Ernst R. Tamm. 2012. "Lack of Endothelial Diaphragms in Fenestrae and Caveolae of Mutant Plvap-Deficient Mice." *Histochemistry and Cell Biology* 138 (5): 709–24. https://doi.org/10.1007/S00418-012-0987-3/FIGURES/9.

Hirayama, Daisuke, Tomoya Iida, and Hiroshi Nakase. 2017. "The Phagocytic Function of Macrophage-Enforcing Innate Immunity and Tissue Homeostasis." *International Journal of Molecular Sciences 2018, Vol. 19, Page 92* 19 (1): 92. https://doi.org/10.3390/IJMS19010092.

Hnasko, R., M. McFarland, and N. Ben-Jonathan. 2002. "Distribution and Characterization of Plasmalemma Vesicle Protein-1 in Rat Endocrine Glands." *The Journal of Endocrinology* 175 (3): 649–61. https://doi.org/10.1677/JOE.0.1750649.

Hnasko, Robert, John Mark Carter, Freddy Medina, Philippe G. Frank, and Michael P. Lisanti. 2006. "PV-1 Labels Trans-Cellular Openings in Mouse Endothelial Cells and Is Negatively Regulated by VEGF." *Cell Cycle* 5 (17): 2021–28. https://doi.org/10.4161/CC.5.17.3217.

Hoeffel, Guillaume, Jinmiao Chen, Yonit Lavin, Donovan Low, Francisca F. Almeida, Peter See, Anna E. Beaudin, et al. 2015. "C-Myb+ Erythro-Myeloid Progenitor-Derived Fetal Monocytes Give Rise to Adult Tissue-Resident Macrophages." *Immunity* 42 (4): 665–78. https://doi.org/10.1016/J.IMMUNI.2015.03.011.

Hoeffel, Guillaume, and Florent Ginhoux. 2018. "Fetal Monocytes and the Origins of Tissue-Resident Macrophages." *Cellular Immunology* 330 (August): 5–15. https://doi.org/10.1016/J.CELLIMM.2018.01.001.

Holash, J. A., S. I. Harik, G. Perry, and P. A. Stewart. 1993. "Barrier Properties of Testis Microvessels." *Proceedings of the National Academy of Sciences of the United States of America* 90 (23): 11069–73. https://doi.org/10.1073/PNAS.90.23.11069.

Hulsmans, Maarten, Sebastian Clauss, Ling Xiao, Aaron D. Aguirre, Kevin R. King, Alan Hanley, William J. Hucker, et al. 2017. "Macrophages Facilitate Electrical Conduction in the Heart." *Cell* 169 (3): 510-522.e20. https://doi.org/10.1016/J.CELL.2017.03.050.

Hume, David A, David Halpintt, Harry Charltont, and Siamon Gordon. 1984. "The Mononuclear Phagocyte System of the Mouse Defined by Immunohistochemical Localization of Antigen F4/80: Macrophages of Endocrine Organs (Pituitary/Adrenal Cortex/Corpus Luteum/Testis)." *Proc. Nal. Acad. Sci. USA* 81: 4174–77. https://www.pnas.org.

Hutson, James C. 1992. "Development of Cytoplasmic Digitations between Leydig Cells and Testicular Macrophages of the Rat." *Cell and Tissue Research* 267 (2): 385–89. https://doi.org/10.1007/BF00302977/METRICS.

Itoh, M., D. G. De Rooij, A. Jansen, and H. A. Drexhage. 1995. "Phenotypical Heterogeneity of Testicular Macrophages/Dendritic Cells in Normal Adult Mice: An Immunohistochemical

Study." *Journal of Reproductive Immunology* 28 (3): 217–32. https://doi.org/10.1016/0165-0378(95)00923-9.

Ivanova, Anna, Massimo Signore, Nadia Caro, Nicholas D.E. Greene, Andrew J. Copp, and Juan Pedro Martinez-Barbera. 2005. "In Vivo Genetic Ablation by Cre-Mediated Expression of Diphtheria Toxin Fragment A." *Genesis (United States)* 43 (3): 129–35. https://doi.org/10.1002/GENE.20162.

Ivanovs, Andrejs, Stanislav Rybtsov, Elizabeth S. Ng, Edouard G. Stanley, Andrew G. Elefanty, and Alexander Medvinsky. 2017. "Human Haematopoietic Stem Cell Development: From the Embryo to the Dish." *Development* 144 (13): 2323–37. https://doi.org/10.1242/DEV.134866.

Iwasaki, Akiko, and Ruslan Medzhitov. 2015. "Control of Adaptive Immunity by the Innate Immune System." *NATURE IMMUNOLOGY* 16. https://doi.org/10.1038/ni.3123.

Jäppinen, Norma, Inês Félix, Emmi Lokka, Sofia Tyystjärvi, Anne Pynttäri, Tiina Lahtela, Heidi Gerke, Kati Elima, Pia Rantakari, and Marko Salmi. 2019. "Fetal-Derived Macrophages Dominate in Adult Mammary Glands." *Nature Communications 2019 10:1* 10 (1): 1–12. https://doi.org/10.1038/s41467-018-08065-1.

Jokela, Heli, Emmi Lokka, Miikka Kiviranta, Sofia Tyystjärvi, Heidi Gerke, Kati Elima, Marko Salmi, and Pia Rantakari. 2020. "Fetal-Derived Macrophages Persist and Sequentially Maturate in Ovaries after Birth in Mice." *European Journal of Immunology* 50 (10): 1500–1514. https://doi.org/10.1002/EJI.202048531.

Jung, Steffen, Julio Aliberti, Petra Graemmel, Mary Jean Sunshine, Georg W. Kreutzberg, Alan Sher, and Dan R. Littman. 2000. " Analysis of Fractalkine Receptor CX 3 CR1 Function by Targeted Deletion and Green Fluorescent Protein Reporter Gene Insertion ." *Molecular and Cellular Biology* 20 (11): 4106–14. https://doi.org/10.1128/MCB.20.11.4106-4114.2000.

Kalucka, Joanna, Laura P.M.H. de Rooij, Jermaine Goveia, Katerina Rohlenova, Sébastien J. Dumas, Elda Meta, Nadine V. Conchinha, et al. 2020. "Single-Cell Transcriptome Atlas of Murine Endothelial Cells." *Cell* 180 (4): 764-779.e20. https://doi.org/10.1016/J.CELL.2020.01.015.

Kayama, Hisako, and Kiyoshi Takeda. 2016. "Functions of Innate Immune Cells and Commensal Bacteria in Gut Homeostasis." *The Journal of Biochemistry* 159 (2): 141–49. https://doi.org/10.1093/JB/MVV119.

Keuschnigg, Johannes, Tiina Henttinen, Kaisa Auvinen, Marika Karikoski, Marko Salmi, and Sirpa Jalkanen. 2009. "The Prototype Endothelial Marker PAL-E Is a Leukocyte Trafficking Molecule." *Blood* 114 (2): 478–84. https://doi.org/10.1182/BLOOD-2008-11-188763.

Keuschnigg, Johannes, Denis Tvorogov, Kati Elima, Marko Salmi, Kari Alitalo, Tiina Salminen, and Sirpa Jalkanen. 2012. "PV-1 Is Recognized by the PAL-E Antibody and Forms Complexes with NRP-1." *Blood* 120 (1): 232–35. https://doi.org/10.1182/BLOOD-2012-01-406876.

Khan, Jalal A., Avital Mendelson, Yuya Kunisaki, Alexander Birbrair, Yan Kou, Anna Arnal-Estapé, Sandra Pinho, et al. 2016. "Fetal Liver Hematopoietic Stem Cell Niches Associate with Portal Vessels." *Science* 351 (6269): 176–80. https://doi.org/10.1126/SCIENCE.AAD0084.

Kim, Hyeonhui, Minki Kim, Sun Kyoung Im, and Sungsoon Fang. 2018. "Mouse Cre-LoxP System: General Principles to Determine Tissue-Specific Roles of Target Genes." *Laboratory Animal Research* 34 (4): 147. https://doi.org/10.5625/LAR.2018.34.4.147.

Koch, Philipp Sebastian, Ki Hong Lee, Sergij Goerdt, and Hellmut G. Augustin. 2021. "Angiodiversity and Organotypic Functions of Sinusoidal Endothelial Cells." *Angiogenesis 2021 24:2* 24 (2): 289–310. https://doi.org/10.1007/S10456-021-09780-Y.

Kokkinaki, Maria, Tin Lap Lee, Zuping He, Jiji Jiang, Nady Golestaneh, Marie Claude Hofmann, Wai Yee Chan, and Martin Dym. 2009. "The Molecular Signature of Spermatogonial Stem/Progenitor Cells in the 6-Day-Old Mouse Testis." *Biology of Reproduction* 80 (4): 707–

17. https://doi.org/10.1095/BIOLREPROD.108.073809.

Krüger-Genge, Anne, Anna Blocki, Ralf Peter Franke, and Friedrich Jung. 2019. "Vascular Endothelial Cell Biology: An Update." *International Journal of Molecular Sciences 2019, Vol. 20, Page 4411* 20 (18): 4411. https://doi.org/10.3390/IJMS20184411.

Kumaravelu, Parasakthy, Lilian Hook, Aline M. Morrison, Jan Ure, Suling Zhao, Sergie Zuyev, John Ansell, and Alexander Medvinsky. 2002. "Quantitative Developmental Anatomy of Definitive Haematopoietic Stem Cells/Long-Term Repopulating Units (HSC/RUs): Role of the Aorta-Gonad-Mesonephros (AGM) Region and the Yolk Sac in Colonisation of the Mouse Embryonic Liver." *Development* 129 (21): 4891–99. https://doi.org/10.1242/DEV.129.21.4891.

Kurolap, Alina, Orly Eshach-Adiv, Claudia Gonzaga-Jauregui, Katya Dolnikov, Adi Mory, Tamar Paperna, Tova Hershkovitz, et al. 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. https://doi.org/10.1136/JMEDGENET-2018-105299.

Laar, Lianne van de, Wouter Saelens, Sofie De Prijck, Liesbet Martens, Charlotte L. Scott, Gert Van Isterdael, Eik Hoffmann, et al. 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. https://doi.org/10.1016/J.IMMUNI.2016.02.017.

Lavin, Yonit, Deborah Winter, Ronnie Blecher-Gonen, Eyal David, Hadas Keren-Shaul, Miriam Merad, Steffen Jung, and Ido Amit. 2014. "Tissue-Resident Macrophage Enhancer Landscapes Are Shaped by the Local Microenvironment." *Cell* 159 (6): 1312–26. https://doi.org/10.1016/J.CELL.2014.11.018.

Lewis, Steven M., Adam Williams, and Stephanie C. Eisenbarth. 2019. "Structure and Function of the Immune System in the Spleen." *Science Immunology* 4 (33). https://doi.org/10.1126/SCIIMMUNOL.AAU6085.

Liu, Zhaoyuan, Yaqi Gu, Svetoslav Chakarov, Camille Bleriot, Immanuel Kwok, Xin Chen, Amanda Shin, et al. 2019. "Fate Mapping via Ms4a3-Expression History Traces Monocyte-Derived Cells." *Cell* 178 (6): 1509-1525.e19. https://doi.org/10.1016/J.CELL.2019.08.009.

Louis, Cynthia, Andrew D. Cook, Derek Lacey, Andrew J. Fleetwood, Ross Vlahos, Gary P. Anderson, and John A. Hamilton. 2015. "Specific Contributions of CSF-1 and GM-CSF to the Dynamics of the Mononuclear Phagocyte System." *The Journal of Immunology* 195 (1): 134–44. https://doi.org/10.4049/JIMMUNOL.1500369.

Lukyanenko, Yevgeniya, Jau Jiin Chen, and James C. Hutson. 2002. "Testosterone Regulates 25-Hydroxycholesterol Production in Testicular Macrophages." *Biology of Reproduction* 67 (5): 1435–38. https://doi.org/10.1095/BIOLREPROD.102.007575.

MacDonald, Kelli P.A., James S. Palmer, Stephen Cronau, Elke Seppanen, Stuart Olver, Neil C. Raffelt, Rachel Kuns, et al. 2010. "An Antibody against the Colony-Stimulating Factor 1 Receptor Depletes the Resident Subset of Monocytes and Tissue- and Tumor-Associated Macrophages but Does Not Inhibit Inflammation." *Blood* 116 (19): 3955–63. https://doi.org/10.1182/BLOOD-2010-02-266296.

Maiorino, Laura, Juliane Daßler-Plenker, Lijuan Sun, and Mikala Egeblad. 2022. "Innate Immunity and Cancer Pathophysiology." *Https://Doi.Org/10.1146/Annurev-Pathmechdis-032221-115501* 17 (January): 425–57. https://doi.org/10.1146/ANNUREV-PATHMECHDIS-032221-115501.

Mak, Ki M., and Da Wi Shin. 2021. "Hepatic Sinusoids versus Central Veins: Structures, Markers, Angiocrines, and Roles in Liver Regeneration and Homeostasis." *The Anatomical Record* 304 (8): 1661–91. https://doi.org/10.1002/AR.24560.

Mäkelä, Juho-Antti, and Robin M Hobbs. 2019. "REPRODUCTION REVIEW Molecular Regulation of Spermatogonial Stem Cell Renewal and Differentiation." https://doi.org/10.1530/REP.

Mäkelä, Juho-Antti, Jaakko J Koskenniemi, Helena E Virtanen, and Jorma Toppari. 2019. “Testis Development.” *Endocrine Reviews* 40 (4): 857–905. https://doi.org/10.1210/er.2018-00140.

Matsumoto, K., H. Yoshitomi, J. Rossant, and K. S. Zaret. 2001. “Liver Organogenesis Promoted by Endothelial Cells Prior to Vascular Function.” *Science* 294 (5542): 559–63. https://doi.org/10.1126/SCIENCE.1063889/SUPPL_FILE/1063889S2_THUMB.GIF.

McBrien, Claire N., and Andrew Menzies-Gow. 2017. “The Biology of Eosinophils and Their Role in Asthma.” *Frontiers in Medicine* 4 (JUN): 269788. https://doi.org/10.3389/FMED.2017.00093/BIBTEX.

McGrath, Kathleen E., Jenna M. Frame, Katherine H. Fegan, James R. Bowen, Simon J. Conway, Seana C. Catherman, Paul D. Kingsley, Anne D. Koniski, and James Palis. 2015. “Distinct Sources of Hematopoietic Progenitors Emerge before HSCs and Provide Functional Blood Cells in the Mammalian Embryo.” *Cell Reports* 11 (12): 1892–1904. https://doi.org/10.1016/j.celrep.2015.05.036.

McGrath, Kathleen E., Jenna M. Frame, and James Palis. 2015. “Early Hematopoiesis and Macrophage Development.” *Seminars in Immunology* 27 (6): 379–87. https://doi.org/10.1016/J.SMIM.2016.03.013.

McLachlan, R. I., L. O’Donnell, S. J. Meachem, P. G. Stanton, D. M. De Kretser, K. Pratis, and D. M. Robertson. 2002. “Identification of Specific Sites of Hormonal Regulation in Spermatogenesis in Rats, Monkeys, and Man.” *Recent Progress in Hormone Research* 57: 149–79. https://doi.org/10.1210/RP.57.1.149.

Medvinsky, Alexander, and Elaine Dzierzak. 1996. “Definitive Hematopoiesis Is Autonomously Initiated by the AGM Region.” *Cell* 86 (6): 897–906. https://doi.org/10.1016/S0092-8674(00)80165-8.

Meng, Xiaojuan, Maria Lindahl, Mervi E. Hyvönen, Martti Parvinen, Dirk G. De Rooij, Michael W. Hess, Anne Raatikainen-Ahokas, et al. 2000. “Regulation of Cell Fate Decision of Undifferentiated Spermatogonia by GDNF.” *Science* 287 (5457): 1489–93. https://doi.org/10.1126/SCIENCE.287.5457.1489/SUPPL_FILE/1046816S5_THUMB.GIF.

Merad, Miriam, Markus G. Manz, Holger Karsunky, Amy Wagers, Wendy Peters, Israel Charo, Irving L. Weissman, Jason G. Cyster, and Edgar G. Engleman. 2002. “Langerhans Cells Renew in the Skin throughout Life under Steady-State Conditions.” *Nature Immunology 2002 3:12* 3 (12): 1135–41. https://doi.org/10.1038/ni852.

Metzger, D., J. Clifford, H. Chiba, and P. Chambon. 1995. “Conditional Site-Specific Recombination in Mammalian Cells Using a Ligand- Dependent Chimeric Cre Recombinase.” *Proceedings of the National Academy of Sciences of the United States of America* 92 (15): 6991–95. https://doi.org/10.1073/PNAS.92.15.6991.

Michel, C.C., and C.R. Neal. 1999. “Openings Through Endothelial Cells Associated with Increased Microvascular Permeability.” *Microcirculation* 6 (1): 45–54. https://doi.org/10.1111/J.1549-8719.1999.TB00086.X.

Mildner, Alexander, Hauke Schmidt, Mirko Nitsche, Doron Merkler, Uwe Karsten Hanisch, Matthias Mack, Mathias Heikenwalder, Wolfgang Brück, Josef Priller, and Marco Prinz. 2007. “Microglia in the Adult Brain Arise from Ly-6ChiCCR2+ Monocytes Only under Defined Host Conditions.” *Nature Neuroscience 2007 10:12* 10 (12): 1544–53. https://doi.org/10.1038/nn2015.

Miyake, Kensuke, Sho Shibata, Soichiro Yoshikawa, and Hajime Karasuyama. 2021. “Basophils and Their Effector Molecules in Allergic Disorders.” *Allergy* 76 (6): 1693–1706. https://doi.org/10.1111/ALL.14662.

Montecino-Rodriguez, Encarnacion, Hyosuk Leathers, and Kenneth Dorshkind. 2006. “Identification of a B-1 B Cell–Specified Progenitor.” *Nature Immunology 2006 7:3* 7 (3): 293–301.

https://doi.org/10.1038/ni1301.

Morris, L., C. F. Graham, and S. Gordon. 1991. “Macrophages in Haemopoietic and Other Tissues of the Developing Mouse Detected by the Monoclonal Antibody F4/80.” *Development* 112 (2): 517–26. https://doi.org/10.1242/DEV.112.2.517.

Mossadegh-Keller, Noushin, Rebecca Gentek, Gregory Gimenez, Sylvain Bigot, Sebastien Mailfert, and Michael H. Sieweke. 2017. “Developmental Origin and Maintenance of Distinct Testicular Macrophage Populations.” *The Journal of Experimental Medicine* 214 (10): 2829–41. https://doi.org/10.1084/jem.20170829.

Mossadegh-Keller, Noushin, and Michael H. Sieweke. 2018. “Testicular Macrophages: Guardians of Fertility.” *Cellular Immunology* 330 (August): 120–25. https://doi.org/10.1016/J.CELLIMM.2018.03.009.

Mruk, Dolores D., and C. Yan Cheng. 2015. “The Mammalian Blood-Testis Barrier: Its Biology and Regulation.” *Endocrine Reviews* 36 (5): 564–91. https://doi.org/10.1210/ER.2014-1101.

Müller, Albrecht M., Alexander Medvinsky, John Strouboulis, Frank Grosveld, and Elaine Dzierzakt. 1994. “Development of Hematopoietic Stem Cell Activity in the Mouse Embryo.” *Immunity* 1 (4): 291–301. https://doi.org/10.1016/1074-7613(94)90081-7.

Nagasawa, Maho, Hergen Spits, and Xavier Romero Ros. 2018. “Innate Lymphoid Cells (ILCs): Cytokine Hubs Regulating Immunity and Tissue Homeostasis.” *Cold Spring Harbor Perspectives in Biology* 10 (12). https://doi.org/10.1101/CSHPERSPECT.A030304.

Naito, Hisamichi, Tomohiro Iba, and Nobuyuki Takakura. 2020. “Mechanisms of New Blood-Vessel Formation and Proliferative Heterogeneity of Endothelial Cells.” *International Immunology* 32 (5): 295–305. https://doi.org/10.1093/INTIMM/DXAA008.

Naito, Makoto, Shin-ichi Hayashi, Hisahiro Yoshida, Shin-ichi Nishikawa, Leonard D Shultz, and Kiyoshi Takahashi. 1991. “Abnormal Differentiation of Tissue Macrophage Populations in ‘Osteopetrosis’ (Op) Mice Defective in the Production of Macrophage Colony-Stimulating Factor.” *American Journal of Patboloy* 139 (3).

Nakagawa, Toshinori, David J. Jörg, Hitomi Watanabe, Seiya Mizuno, Seungmin Han, Tatsuro Ikeda, Yoshiki Omatsu, et al. 2021. “A Multistate Stem Cell Dynamics Maintains Homeostasis in Mouse Spermatogenesis.” *Cell Reports* 37 (3): 109875. https://doi.org/10.1016/J.CELREP.2021.109875.

Nes, W. David, Yevgeniya O. Lukyanenko, Zhong Hua Jia, Stéphane Quideau, William N. Howald, Thomas K. Pratum, Robert R. West, and James C. Hutson. 2000. “Identification of the Lipophilic Factor Produced by Macrophages That Stimulates Steroidogenesis.” *Endocrinology* 141 (3): 953–58. https://doi.org/10.1210/ENDO.141.3.7350.

Niemelä, Harri, Kati Elima, Tiina Henttinen, Heikki Irjala, Marko Salmi, and Sirpa Jalkanen. 2005. “Molecular Identification of PAL-E, a Widely Used Endothelial-Cell Marker.” *Blood* 106 (10): 3405–9. https://doi.org/10.1182/BLOOD-2005-01-0254.

Nishimura, Hitoshi, and Steven W. L’Hernault. 2017. “Spermatogenesis.” *Current Biology* 27 (18): R988–94. https://doi.org/10.1016/J.CUB.2017.07.067.

Oatley, Jon M., Melissa J. Oatley, Mary R. Avarbock, John W. Tobias, and Ralph L. Brinster. 2009. “Colony Stimulating Factor 1 Is an Extrinsic Stimulator of Mouse Spermatogonial Stem Cell Self-Renewal.” *Development* 136 (7): 1191–99. https://doi.org/10.1242/DEV.032243.

Ottersbach, Katrin, and Elaine Dzierzak. 2005. “The Murine Placenta Contains Hematopoietic Stem Cells within the Vascular Labyrinth Region.” *Developmental Cell* 8 (3): 377–87. https://doi.org/10.1016/J.DEVCEL.2005.02.001.

Palis, James, Scott Robertson, Marion Kennedy, Charles Wall, and Gordon Keller. 1999. “Development of Erythroid and Myeloid Progenitors in the Yolk Sac and Embryo Proper of the Mouse.”

Development 126 (22): 5073–84. https://doi.org/10.1242/DEV.126.22.5073.

Pelt, A. M.M. Van, and D. G. De Rooij. 1990. "Synchronization of the Seminiferous Epithelium after Vitamin A Replacement in Vitamin A-Deficient Mice." *Biology of Reproduction* 43 (3): 363–67. https://doi.org/10.1095/BIOLREPROD43.3.363.

Poisson, Johanne, Sara Lemoinne, Chantal Boulanger, François Durand, Richard Moreau, Dominique Valla, and Pierre Emmanuel Rautou. 2017. "Liver Sinusoidal Endothelial Cells: Physiology and Role in Liver Diseases." *Journal of Hepatology* 66 (1): 212–27. https://doi.org/10.1016/J.JHEP.2016.07.009.

Ráki, Melinda, Stig Tollefsen, Øyvind Molberg, Knut E.A. Lundin, Ludvig M. Sollid, and Frode L. Jahnsen. 2006. "A Unique Dendritic Cell Subset Accumulates in the Celiac Lesion and Efficiently Activates Gluten-Reactive T Cells." *Gastroenterology* 131 (2): 428–38. https://doi.org/10.1053/J.GASTRO.2006.06.002.

Rantakari, Pia, Kaisa Auvinen, Norma Jäppinen, Maria Kapraali, Joona Valtonen, Marika Karikoski, Heidi Gerke, et al. 2015. "The Endothelial Protein PLVAP in Lymphatics Controls the Entry of Lymphocytes and Antigens into Lymph Nodes." *Nature Immunology 2015 16:4* 16 (4): 386–96. https://doi.org/10.1038/ni.3101.

Razani, Babak, Jeffery A. Engelman, Xiao Bo Wang, William Schubert, Xiao Lan Zhang, Carolyn B. Marks, Frank Macalusol, et al. 2001. "Caveolin-1 Null Mice Are Viable but Show Evidence of Hyperproliferative and Vascular Abnormalities *." *Journal of Biological Chemistry* 276 (41): 38121–38. https://doi.org/10.1074/JBC.M105408200.

Ribatti, Domenico. 2006. "Genetic and Epigenetic Mechanisms in the Early Development of the Vascular System." *Journal of Anatomy* 208 (2): 139–52. https://doi.org/10.1111/J.1469-7580.2006.00522.X.

Rooijen, Nico van, and Esther Hendrikx. 2010. "Liposomes for Specific Depletion of Macrophages from Organs and Tissues." *Methods in Molecular Biology (Clifton, N.J.)* 605: 189–203. https://doi.org/10.1007/978-1-60327-360-2_13/COVER.

Samokhvalov, Igor M., Natalia I. Samokhvalova, and Shin Ichi Nishikawa. 2007. "Cell Tracing Shows the Contribution of the Yolk Sac to Adult Haematopoiesis." *Nature 2006 446:7139* 446 (7139): 1056–61. https://doi.org/10.1038/nature05725.

Sauter, Kristin A., Clare Pridans, Anuj Sehgal, Yi Ting Tsai, Barry M. Bradford, Sobia Raza, Lindsey Moffat, et al. 2014. "Pleiotropic Effects of Extended Blockade of CSF1R Signaling in Adult Mice." *Journal of Leukocyte Biology* 96 (2): 265–74. https://doi.org/10.1189/JLB.2A0114-006R.

Sawai, Catherine M., Sonja Babovic, Samik Upadhaya, David J.H.F. Knapp, Yonit Lavin, Colleen M. Lau, Anton Goloborodko, et al. 2016. "Hematopoietic Stem Cells Are the Major Source of Multilineage Hematopoiesis in Adult Animals." *Immunity* 45 (3): 597–609. https://doi.org/10.1016/J.IMMUNI.2016.08.007.

Schlingemann, R.O., G.M. Dingjan, J.J. Emeis, J. Blok, S.O. Warnaar, and Ruiter D.J. 1985. "Monoclonal Antibody PAL-E Specific for Endothelium." *Lab. Invest.* 52 (1): 71–76. moz-extension://6b154034-ba40-4f90-b1af-153cd0147e47/enhanced-reader.html?openApp&pdf=https%3A%2F%2Frepository.ubn.ru.nl%2Fbitstream%2Fhandle%2F2066%2F113887%2Fmmubn000001_114773793.pdf%3Fsequence%3D1%23page%3D73.

Schneider, Christoph, Samuel P. Nobs, Michael Kurrer, Hubert Rehrauer, Christoph Thiele, and Manfred Kopf. 2014. "Induction of the Nuclear Receptor PPAR-γ by the Cytokine GM-CSF Is Critical for the Differentiation of Fetal Monocytes into Alveolar Macrophages." *Nature Immunology 2014 15:11* 15 (11): 1026–37. https://doi.org/10.1038/ni.3005.

Schubert, William, Philippe G. Frank, Babak Razani, David S. Park, Chi Wing Chow, and Michael P.

Lisanti. 2001. “Caveolae-Deficient Endothelial Cells Show Defects in the Uptake and Transport of Albumin in Vivo.” *Journal of Biological Chemistry* 276 (52): 48619–22. https://doi.org/10.1074/JBC.C100613200.

Schulz, Christian, Elisa Gomez Perdiguero, Laurent Chorro, Heather Szabo-Rogers, Nicolas Cagnard, Katrin Kierdorf, Marco Prinz, et al. 2012. “A Lineage of Myeloid Cells Independent of Myb and Hematopoietic Stem Cells.” *Science*, April. https://doi.org/10.1126/SCIENCE.1219179.

Scott, Charlotte L., Fang Zheng, Patrick De Baetselier, Liesbet Martens, Yvan Saeys, Sofie De Prijck, Saskia Lippens, et al. 2016. “Bone Marrow-Derived Monocytes Give Rise to Self-Renewing and Fully Differentiated Kupffer Cells.” *Nature Communications 2015 7:1* 7 (1): 1–10. https://doi.org/10.1038/ncomms10321.

Serbina, Natalya V., and Eric G. Pamer. 2006. “Monocyte Emigration from Bone Marrow during Bacterial Infection Requires Signals Mediated by Chemokine Receptor CCR2.” *Nature Immunology 2006 7:3* 7 (3): 311–17. https://doi.org/10.1038/ni1309.

Seternes, Tore, Karen Sørensen, and Bård Smedsrød. 2002. “Scavenger Endothelial Cells of Vertebrates: A Nonperipheral Leukocyte System for High-Capacity Elimination of Waste Macromolecules.” *Proceedings of the National Academy of Sciences of the United States of America* 99 (11): 7594–97. https://doi.org/10.1073/PNAS.102173299/ASSET/97FBE5A1-4E15-4AF0-BD0D-5C04F27EB575/ASSETS/GRAPHIC/PQ1021732002.JPEG.

Shechter, Ravid, Anat London, and Michal Schwartz. 2013. “Orchestrated Leukocyte Recruitment to Immune-Privileged Sites: Absolute Barriers versus Educational Gates.” *Nature Reviews Immunology 2013 13:3* 13 (3): 206–18. https://doi.org/10.1038/nri3391.

Sheng, Jianpeng, Christiane Ruedl, and Klaus Karjalainen. 2015. “Most Tissue-Resident Macrophages Except Microglia Are Derived from Fetal Hematopoietic Stem Cells.” *Immunity* 43 (2): 382–93. https://doi.org/10.1016/J.IMMUNI.2015.07.016.

Short, Christie, Hong K. Lim, Jonathan Tan, and Helen C. O’Neill. 2019. “Targeting the Spleen as an Alternative Site for Hematopoiesis.” *BioEssays* 41 (5): 1800234. https://doi.org/10.1002/BIES.201800234.

Shue, Eveline H., Eleanor B. Carson-Walter, Yang Liu, Bethany N. Winans, Zarina S. Ali, Jun Chen, and Kevin A. Walter. 2008. “Plasmalemmal Vesicle Associated Protein-1 (PV-1) Is a Marker of Blood-Brain Barrier Disruption in Rodent Models.” *BMC Neuroscience* 9 (1): 1–9. https://doi.org/10.1186/1471-2202-9-29/FIGURES/4.

Sierro, Frederic, Maximilien Evrard, Simone Rizzetto, Michelle Melino, Andrew J. Mitchell, Manuela Florido, Lynette Beattie, et al. 2017. “A Liver Capsular Network of Monocyte-Derived Macrophages Restricts Hepatic Dissemination of Intraperitoneal Bacteria by Neutrophil Recruitment.” *Immunity* 47 (2): 374-388.e6. https://doi.org/10.1016/J.IMMUNI.2017.07.018.

Simons, Michael, Emma Gordon, and Lena Claesson-Welsh. 2016. “Mechanisms and Regulation of Endothelial VEGF Receptor Signalling.” *Nature Reviews Molecular Cell Biology 2016 17:10* 17 (10): 611–25. https://doi.org/10.1038/nrm.2016.87.

Soares-Da-Silva, Francisca, Laina Freyer, Ramy Elsaid, Odile Burlen-Defranoux, Lorea Iturri, Odile Sismeiro, Perpétua Pinto-Do, Elisa Gomez-Perdiguero, and Ana Cumano. 2021. “Yolk Sac, but Not Hematopoietic Stem Cell–Derived Progenitors, Sustain Erythropoiesis throughout Murine Embryonic Life.” *Journal of Experimental Medicine* 218 (4). https://doi.org/10.1084/JEM.20201729/211777.

Söderberg, Ola, Mats Gullberg, Malin Jarvius, Karin Ridderstråle, Karl Johan Leuchowius, Jonas Jarvius, Kenneth Wester, et al. 2006. “Direct Observation of Individual Endogenous Protein Complexes in Situ by Proximity Ligation.” *Nature Methods 2006 3:12* 3 (12): 995–1000. https://doi.org/10.1038/nmeth947.

Spadoni, Ilaria, Elena Zagato, Alice Bertocchi, Roberta Paolinelli, Edina Hot, Antonio Di Sabatino, Flavio Caprioli, et al. 2015. “A Gut-Vascular Barrier Controls the Systemic Dissemination of Bacteria.” *Science* 350 (6262): 830–34. https://doi.org/10.1126/SCIENCE.AAD0135/SUPPL_FILE/AAD0135S6.MOV.

Squarzoni, Paola, Guillaume Oller, Guillaume Hoeffel, Lorena Pont-Lezica, Philippe Rostaing, Donovan Low, Alain Bessis, Florent Ginhoux, and Sonia Garel. 2014. “Microglia Modulate Wiring of the Embryonic Forebrain.” *Cell Reports* 8 (5): 1271–79. https://doi.org/10.1016/J.CELREP.2014.07.042.

Srinivas, Shankar, Tomoko Watanabe, Chyuan Sheng Lin, Chris M. William, Yasuto Tanabe, Thomas M. Jessell, and Frank Costantini. 2001. “Cre Reporter Strains Produced by Targeted Insertion of EYFP and ECFP into the ROSA26 Locus.” *BMC Developmental Biology* 1 (1): 1–8. https://doi.org/10.1186/1471-213X-1-4/FIGURES/4.

Stan, Lucian Ghitescu, Bruce S. Jacobson, and George E. Palade. 1999. “Isolation, Cloning, and Localization of Rat PV-1, a Novel Endothelial Caveolar Protein.” *The Journal of Cell Biology* 145 (6): 1189. https://doi.org/10.1083/JCB.145.6.1189.

Stan, Kubitza, and Palade. 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. https://doi.org/10.1073/PNAS.96.23.13203/ASSET/E32A4BE5-8DA2-4E07-A65E-B64893DD84FF/ASSETS/GRAPHIC/PQ2394025003.JPEG.

Stan, R. V. 2007. “Endothelial Stomatal and Fenestral Diaphragms in Normal Vessels and Angiogenesis.” *Journal of Cellular and Molecular Medicine* 11 (4): 621–43. https://doi.org/10.1111/J.1582-4934.2007.00075.X.

Stan, Radu Virgil, Karen C. Arden, and George E. Palade. 2001. “CDNA and Protein Sequence, Genomic Organization, and Analysis of Cis Regulatory Elements of Mouse and Human PLVAP Genes.” *Genomics* 72 (3): 304–13. https://doi.org/10.1006/GENO.2000.6489.

Stan, Tkachenko, and Niesman. 2004. “PV1 Is a Key Structural Component for the Formation of the Stomatal and Fenestral Diaphragms.” *Molecular Biology of the Cell* 15 (8): 3615–30. https://doi.org/10.1091/MBC.E03-08-0593/ASSET/IMAGES/LARGE/ZMK0080427650010.JPEG.

Stan, Dan Tse, Sophie J. Deharvengt, Nicole C. Smits, Yan Xu, Marcus R. Luciano, Caitlin L. McGarry, et al. 2012. “The Diaphragms of Fenestrated Endothelia: Gatekeepers of Vascular Permeability and Blood Composition.” *Developmental Cell* 23 (6): 1203–18. https://doi.org/10.1016/J.DEVCEL.2012.11.003.

Stolen, Craig M., Fumiko Marttila-Ichihara, Kaisa Koskinen, Gennady G. Yegutkin, Raisa Turja, Petri Bono, Mikael Skurnik, Arno Hänninen, Sirpa Jalkanen, and Marko Salmi. 2005. “Absence of the Endothelial Oxidase AOC3 Leads to Abnormal Leukocyte Traffic in Vivo.” *Immunity* 22 (1): 105–15. https://doi.org/10.1016/J.IMMUNI.2004.12.006.

Strickland, Laura A., Adrian M. Jubb, Jo Anne Hongo, Fiona Zhong, Jennifer Burwick, Ling Fu, Gretchen D. Frantz, and Hartmut Koeppen. 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. https://doi.org/10.1002/PATH.1805.

Sunderkötter, Cord, Tatjana Nikolic, Marilyn J. Dillon, Nico van Rooijen, Martin Stehling, Douglas A. Drevets, and Pieter J. M. Leenen. 2004. “Subpopulations of Mouse Blood Monocytes Differ in Maturation Stage and Inflammatory Response.” *The Journal of Immunology* 172 (7): 4410–17. https://doi.org/10.4049/JIMMUNOL.172.7.4410.

Suzuki, Takuji, Takuro Sakagami, Bruce K. Rubin, Lawrence M. Nogee, Robert E. Wood, Sarah L.

Zimmerman, Teresa Smolarek, et al. 2008. “Familial Pulmonary Alveolar Proteinosis Caused by Mutations in CSF2RA.” *Journal of Experimental Medicine* 205 (12): 2703–10. https://doi.org/10.1084/JEM.20080990.

Tacke, Robert, Ingo Hilgendorf, Hannah Garner, Claire Waterborg, Kiwon Park, Heba Nowyhed, Richard N. Hanna, et al. 2015. “The Transcription Factor NR4A1 Is Essential for the Development of a Novel Macrophage Subset in the Thymus.” *Scientific Reports 2015 5:1* 5 (1): 1–13. https://doi.org/10.1038/srep10055.

Tall, Alan R., and Laurent Yvan-Charvet. 2015. “Cholesterol, Inflammation and Innate Immunity.” *Nature Reviews Immunology 2015 15:2* 15 (2): 104–16. https://doi.org/10.1038/nri3793.

Terkelsen, Mike K., Sofie M. Bendixen, Daniel Hansen, Emma A.H. Scott, Andreas F. Moeller, Ronni Nielsen, Susanne Mandrup, et al. 2020. “Transcriptional Dynamics of Hepatic Sinusoid-Associated Cells After Liver Injury.” *Hepatology* 72 (6): 2119–33. https://doi.org/10.1002/HEP.31215.

Thompson, Linda F., Holger K. Eltzschig, Juan C. Ibla, C. Justin Van De Wiele, Regina Resta, Julio C. Morote-Garcia, and Sean P. Colgan. 2004. “Crucial Role for Ecto-5′-Nucleotidase (CD73) in Vascular Leakage during Hypoxia.” *Journal of Experimental Medicine* 200 (11): 1395–1405. https://doi.org/10.1084/JEM.20040915.

Tkachenko, Eugene, Dan Tse, Olga Sideleva, Sophie J. Deharvengt, Marcus R. Luciano, Yan Xu, Caitlin L. McGarry, et al. 2012. “Caveolae, Fenestrae and Transendothelial Channels Retain PV1 on the Surface of Endothelial Cells.” *PLOS ONE* 7 (3): e32655. https://doi.org/10.1371/JOURNAL.PONE.0032655.

Tober, Joanna, Anne Koniski, Kathleen E. McGrath, Radhika Vemishetti, Rachael Emerson, Karen K.L. De Mesy-Bentley, Richard Waugh, and James Palis. 2007. “The Megakaryocyte Lineage Originates from Hemangioblast Precursors and Is an Integral Component Both of Primitive and of Definitive Hematopoiesis.” *Blood* 109 (4): 1433–41. https://doi.org/10.1182/BLOOD-2006-06-031898.

Tompkins, Andrew B., Paul Hutchinson, David M. De Kretser, and Mark P. Hedger. 1998. “Characterization of Lymphocytes in the Adult Rat Testis by Flow Cytometry: Effects Of Activin and Transforming Growth Factor β on Lymphocyte Subsets in Vitro.” *Biology of Reproduction* 58 (4): 943–51. https://doi.org/10.1095/BIOLREPROD58.4.943.

Turvey, Stuart E., and David H. Broide. 2010. “Innate Immunity.” *Journal of Allergy and Clinical Immunology* 125 (2): S24–32. https://doi.org/10.1016/J.JACI.2009.07.016.

Varricchi, Gilda, Francesca Wanda Rossi, Maria Rosaria Galdiero, Francescopaolo Granata, Gjada Criscuolo, Giuseppe Spadaro, Amato De Paulis, and Gianni Marone. 2019. “Physiological Roles of Mast Cells: Collegium Internationale Allergologicum Update 2019.” *International Archives of Allergy and Immunology* 179 (4): 247–61. https://doi.org/10.1159/000500088.

Vergouwen, R. P.F.A., S. G.P.M. Jacobs, R. Huiskamp, J. A.G. Davids, and D. G. De Rooij. 1991. “Proliferative Activity of Gonocytes, Sertoli Cells and Interstitial Cells during Testicular Development in Mice.” *Reproduction* 93 (1): 233–43. https://doi.org/10.1530/JRF.0.0930233.

Vivier, Eric, David Artis, Marco Colonna, Andreas Diefenbach, James P. Di Santo, Gérard Eberl, Shigeo Koyasu, et al. 2018. “Innate Lymphoid Cells: 10 Years On.” *Cell* 174 (5): 1054–66. https://doi.org/10.1016/J.CELL.2018.07.017.

Wakabayashi, Taku, Hisamichi Naito, Jun ichi Suehiro, Yang Lin, Hideya Kawaji, Tomohiro Iba, Tsukasa Kouno, et al. 2018. “CD157 Marks Tissue-Resident Endothelial Stem Cells with Homeostatic and Regenerative Properties.” *Cell Stem Cell* 22 (3): 384-397.e6. https://doi.org/10.1016/J.STEM.2018.01.010.

Wang, Ming, Yalong Yang, Dilay Cansever, Yiming Wang, Crystal Kantores, Sébastien Messiaen,

Delphine Moison, et al. 2021. “Two Populations of Self-Maintaining Monocyte-Independent Macrophages Exist in Adult Epididymis and Testis.” *Proceedings of the National Academy of Sciences of the United States of America* 118 (1): e2013686117. https://doi.org/10.1073/PNAS.2013686117/SUPPL_FILE/PNAS.2013686117.SD01.XLSX.

Wei, Quxing, Yanyue Deng, Qianqian Yang, Angyu Zhan, and Lexun Wang. 2023. “The Markers to Delineate Different Phenotypes of Macrophages Related to Metabolic Disorders.” *Frontiers in Immunology* 14 (February). https://doi.org/10.3389/FIMMU.2023.1084636.

Wiktor-Jedrzejczak, Wieslaw, Anna Bartocci, Anthony W. Ferrante, Aftab Ahmed-Ansari, Kenneth W. Sell, Jeffrey W. Pollard, and E. Richard Stanley. 1990. “Total Absence of Colony-Stimulating Factor 1 in the Macrophage-Deficient Osteopetrotic (Op/Op) Mouse.” *Proceedings of the National Academy of Sciences of the United States of America* 87 (12): 4828–32. https://doi.org/10.1073/PNAS.87.12.4828.

Wisse, E. 1970. “An Electron Microscopic Study of the Fenestrated Endothelial Lining of Rat Liver Sinusoids.” *Journal of Ultrasructure Research* 31 (1–2): 125–50. https://doi.org/10.1016/S0022-5320(70)90150-4.

Xie, Yanshe, Changhua Wu, Zicong Li, Zhenfang Wu, and Linjun Hong. 2022. “Early Gonadal Development and Sex Determination in Mammal.” *International Journal of Molecular Sciences 2022, Vol. 23, Page 7500* 23 (14): 7500. https://doi.org/10.3390/IJMS23147500.

Yokomizo, Tomomasa, Takako Ideue, Saori Morino-Koga, Cheng Yong Tham, Tomohiko Sato, Naoki Takeda, Yoshiaki Kubota, et al. 2022. “Independent Origins of Fetal Liver Haematopoietic Stem and Progenitor Cells.” *Nature 2022 609:7928* 609 (7928): 779–84. https://doi.org/10.1038/s41586-022-05203-0.

Yokomizo, Tomomasa, Tomoko Yamada-Inagawa, Amanda D. Yzaguirre, Michael J. Chen, Nancy A. Speck, and Elaine Dzierzak. 2012. “Whole-Mount Three-Dimensional Imaging of Internally Localized Immunostained Cells within Mouse Embryos.” *Nature Protocols 2012 7:3* 7 (3): 421–31. https://doi.org/10.1038/nprot.2011.441.

Yona, Simon, Ki Wook Kim, Yochai Wolf, Alexander Mildner, Diana Varol, Michal Breker, Dalit Strauss-Ayali, et al. 2013. “Fate Mapping Reveals Origins and Dynamics of Monocytes and Tissue Macrophages under Homeostasis.” *Immunity* 38 (1): 79–91. https://doi.org/10.1016/J.IMMUNI.2012.12.001.

Yoshida, Shosei, Mamiko Sukeno, and Yo Ichi Nabeshima. 2007. “A Vasculature-Associated Niche for Undifferentiated Spermatogonia in the Mouse Testis.” *Science* 317 (5845): 1722–26. https://doi.org/10.1126/SCIENCE.1144885/SUPPL_FILE/YOSHIDA-SOM.PDF.

Yoshimoto, Momoko, Encarnacion Montecino-Rodriguez, Michael J. Ferkowicz, Prashanth Porayette, W. Christopher Shelley, Simon J. Conway, Kenneth Dorshkind, and Mervin C. Yoder. 2011. “Embryonic Day 9 Yolk Sac and Intra-Embryonic Hemogenic Endothelium Independently Generate a B-1 and Marginal Zone Progenitor Lacking B-2 Potential.” *Proceedings of the National Academy of Sciences of the United States of America* 108 (4): 1468–73. https://doi.org/10.1073/PNAS.1015841108/SUPPL_FILE/PNAS.201015841SI.PDF.

Zeng, Qun, Mira Mousa, Aisha Shigna Nadukkandy, Lies Franssens, Halima Alnaqbi, Fatima Yousif Alshamsi, Habiba Al Safar, and Peter Carmeliet. 2023. “Understanding Tumour Endothelial Cell Heterogeneity and Function from Single-Cell Omics.” *Nature Reviews Cancer 2023*, June, 1–21. https://doi.org/10.1038/s41568-023-00591-5.

Zhang, Yi, Kathleen E. McGrath, Edward Ayoub, Paul D. Kingsley, Hongbo Yu, Kate Fegan, Kelly A. McGlynn, Sarah Rudzinskas, James Palis, and Archibald S. Perkins. 2021. “Mds1CreERT2, an Inducible Cre Allele Specific to Adult-Repopulating Hematopoietic Stem Cells.” *Cell Reports* 36 (7): 109562. https://doi.org/10.1016/J.CELREP.2021.109562.

Zhao, Shutao, Weiwei Zhu, Shepu Xue, and Daishu Han. 2014. “Testicular Defense Systems: Immune Privilege and Innate Immunity.” *Cellular & Molecular Immunology 2014 11:5* 11 (5): 428–37. https://doi.org/10.1038/cmi.2014.38.

Zhou, Xu, Ruth A. Franklin, Miri Adler, Jeremy B. Jacox, Will Bailis, Justin A. Shyer, Richard A. Flavell, Avi Mayo, Uri Alon, and Ruslan Medzhitov. 2018. “Circuit Design Features of a Stable Two-Cell System.” *Cell* 172 (4): 744-757.e17. https://doi.org/10.1016/J.CELL.2018.01.015.

Zhou, Yulian, Yanshu Wang, Max Tischfield, John Williams, Philip M. Smallwood, Amir Rattner, Makoto M. Taketo, and Jeremy Nathans. 2014. “Canonical WNT Signaling Components in Vascular Development and Barrier Formation.” *The Journal of Clinical Investigation* 124 (9): 3825–46. https://doi.org/10.1172/JCI76431.

Zirkin, Barry R., and Vassilios Papadopoulos. 2018. “Leydig Cells: Formation, Function, and Regulation.” *Biology of Reproduction* 99 (1): 101–11. https://doi.org/10.1093/BIOLRE/IOY059.

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