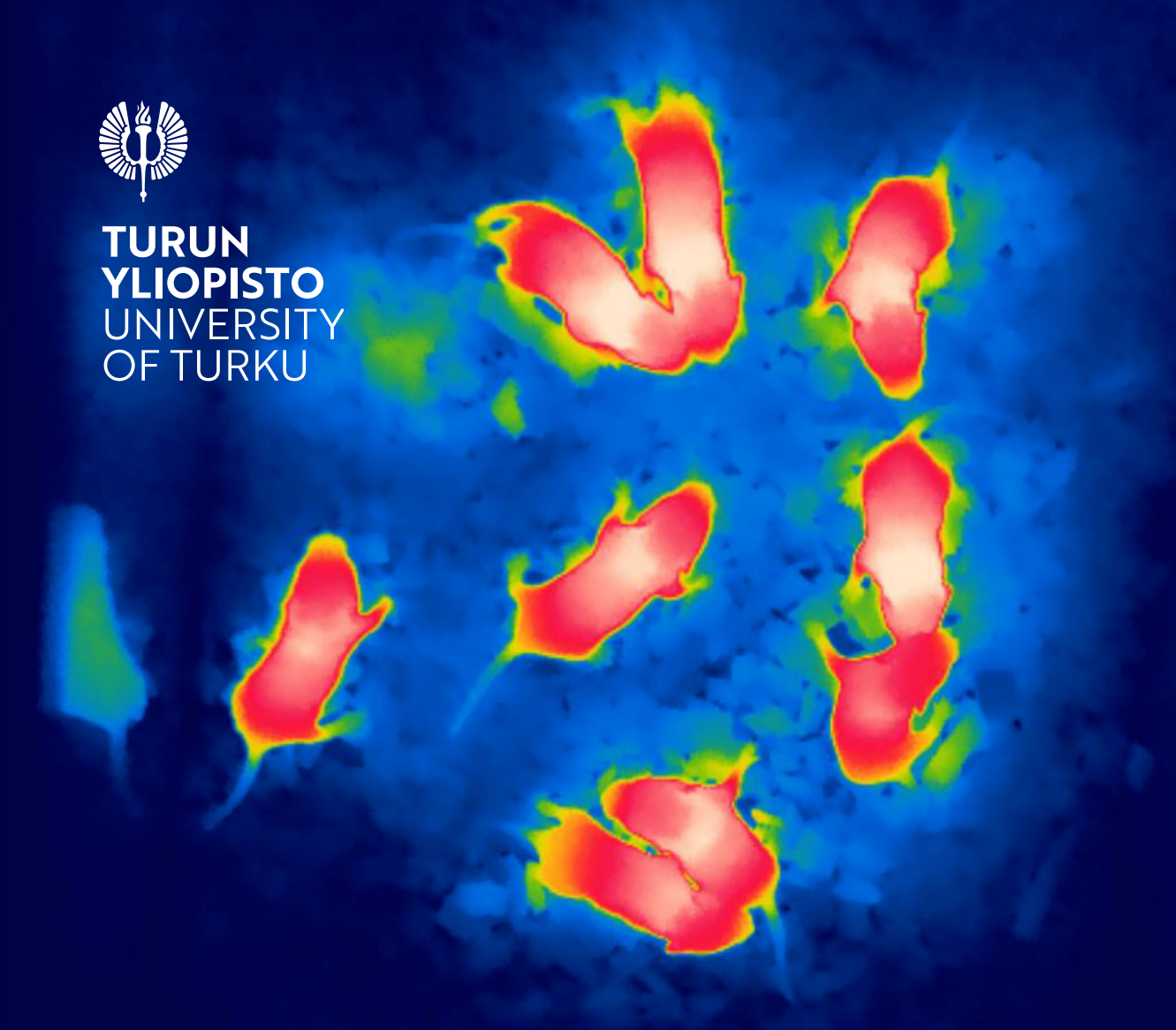

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Inês Alvito Félix

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ABSTRACT

Macrophages are immune cells that also participate in the development and homeostasis of most tissues. Macrophages can originate during embryonic development, first from the yolk sac and later from the fetal liver. Around birth, the bone marrow also starts producing macrophages that can populate the tissues. Tissue resident macrophages have specific functions, responding to cell interactions and local stimuli within the tissue. Most adult resident macrophages are embryonic-derived as these cells are able to self-maintain throughout aging.

Different fat deposits also known as fat pads, exist within the body and may have specific functions. The mammary gland (MG) fat pad is essential for the development of the whole gland, limiting the growth of the ductal network. In mice, epididymal white adipose tissue (eWAT) acts as a cushion for the internal organs, also providing them with energy. Brown adipose tissue (BAT) is a thermogenic organ essential for maintaining the body's core temperature, especially during the perinatal stage. Adipose tissue resident macrophages have been implicated in the development of different pathologies, such as breast neoplasia and obesity. The study of adipose tissue resident macrophages in a developmental steady state may unravel new targets for treating or preventing these pathologies.

This thesis aims to understand the ontogeny and roles of tissue resident macrophages in the development and function of MG, eWAT, and interscapular (i)BAT.

The results show that adult MG resident macrophages are mainly from an embryonic origin, and are essential for the development of the ductal branches of the gland. In eWAT, the embryonic-derived macrophages maintained steady numbers throughout different dietary challenges, suggesting a function in tissue homeostasis for these macrophages. Alternately, iBAT embryonic-derived macrophages are readily replaced by bone marrow-derived macrophages during the first weeks of life. Nevertheless, these macrophages may have a key role in perinatal thermogenesis. Overall, embryonic-derived macrophages seem to have a role in the normal development of different adipose tissues, having specific functions and kinetics according to the fat pad they populate.

KEYWORDS: Macrophages, mammary gland, white adipose tissue, brown adipose tissue, development, obesity

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TIIVISTELMÄ

Makrofagit ovat elimistön puolustusjärjestelmän soluja, jotka osallistuvat myös kudosten kehitykseen ja ylläpitoon. Makrofagit voivat olla peräisin alkionkehityksen ajalta, ensin ruskuaispussista ja myöhemmin alkion maksasta.Vasta syntymän aikoihin luuydin alkaa tuottaa makrofageja kudoksiin. Kudoskohtaisten makrofagien tarkat tehtävät määräytyvät kulloinkin kudoksessa vallitsevien solujen välisten ja paikallisten signaalien mukaan. Suuri osa aikuisen kudoskohtaisista makrofageista on peräisin alkiokaudelta ja ne säilyvät kudoksissa yksilön ikääntyessä.

Elimistön eri rasvakudoksilla on omat tehtävänsä. Rintarauhasten vaalea rasvakudos on välttämätön rintojen kehitykselle, koska se säätelee rintarauhasten kasvua. Hiiren sukupuolirauhasten valkoinen rasvakudos suojaa sisäelimiä ja tarjoaa niille energiaa. Ruskea rasvakudos tuottaa lämpöä ja ylläpitää ruumiinlämpöä etenkin vastasyntyneillä. Rasvakudoksen kudoskohtaiset makrofagit on yhdistetty erilaisten sairauksien, kuten rintasyövän ja liikalihavuuden syntyyn. Rasvakudosten kudoskohtaisten makrofagien tutkimus kehityksen aikana ja normaalitilassa voi paljastaa uusia lääkekehityskohteita näiden sairauksien hoitoon ja ennaltaehkäisyyn.

Tämän väitöskirjatyön tavoitteena oli ymmärtää kudoskohtaisten makrofagien alkuperä ja tehtävät rintarauhasten ja sukupuolirauhasten valkoisen, ja lapaluiden välisen ruskean rasvakudoksen kehityksessä ja toiminnassa.

Tulosten mukaan aikuisen rintarauhasten kudoskohtaiset makrofagit ovat pääasiassa alkioperäisiä ja oleellisia rintatiehyiden haarautumiselle rauhasten kehittyessä. Sukupuolirauhasten rasvakudoksessa alkioperäisten makrofagien määrä pysyy samana erilaisten ruokavalioiden aiheuttaman lihavuuden aikana, mikä viittaa kyseisen solupopulaation konservoituneeseen tehtävään kudoksen ylläpidossa. Lapaluiden välisessä ruskeassa rasvassa alkioperäiset makrofagit korvautuvat luuytimen makrofageilla jo ensimmäisten elinviikkojen aikana. Tästä huolimatta alkioperäisillä makrofageilla on mahdollisesti tärkeä tehtävä vastasyntyneen lämmöntuotannossa. Kaiken kaikkiaan alkioperäisillä makrofageilla näyttää olevan rooli eri rasvakudosten normaalissa kehityksessä, mutta näiden makrofagien tehtävät ja kinetiikka ovat yksilöllisiä jokaisessa tutkitussa rasvakudoksessa.

AVAINSANAT: Makrofagit, rintarauhanen, valkoinen rasvakudos, ruskea rasvakudos, yksilönkehitys, liikalihavuus

Table of Contents

Abbreviations

AGM	Aorta-gonad-mesonephro
APCs	Antigen-presenting cells
ATMs	Adipose tissue resident macrophages
ATP	Adenosine triphosphate
BAT	Brown adipose tissue
BrdU	Bromodeoxyuridine
C/EBPs	CCAAT-enhancer-binding proteins
CCL2	C-C chemokine ligand type 2
CCR2	C-C chemokine receptor type 2
CD	Cluster of differentiation
Chow	Standard diet
CLP	Common lymphoid progenitor
CMoPS	Common monocyte progenitor
CMP	Common myeloid progenitor
Cre-ER	Cyclization recombination - estrogen receptor
CSF1	Macrophage colony stimulating factor 1
CSF1R	Colony stimulating factor 1 receptor
CX3CR1	C-X3-C motif chemokine receptor 1
CXCL14	C-X-C motif chemokine ligand-14
CyTOF	Cytometry by time-of-flight
DEG	Differentially expressed genes
DIO2	Iodothyronine Deiodinase 2
E(15.5)	Embryonic day
ECi	Ethyl cinnamate
EGFP	Enhanced green fluorescent protein
EMPs	Erythromyeloid progenitors
eWAT	Epididymal white adipose tissue
F4/80	coded by adhesion G protein-coupled receptor E1 gene – *ADGRE1*
FACS	Fluorescent activated cell sorting
FC receptor	Fragment crystallizable region receptor
FGF21	Fibroblast growth factor 21
GDF15	Growth and differentiation factor 15
GLUT4	Glucose transporter type 4
GMP	Granulocyte and macrophage progenitor

GTP	Guanosine triphosphate
HFD	High fat diet
HFD+Met	High fat diet with metformin
HSC	Hematopoietic stem cell
HSP	Heat shock proteins
i.p.	Intraperitoneal
i.v.	Intravenous
iBAT	Interscapular brown adipose tissue
IC	Intracellular
IFNγ	Interferon gamma
IL	Interleukine
LAMs	Lipid associated macrophages
LoxP	Locus of crossing [x-ing]-over of bacteriophage P1
LPS	Lipopolysaccharides
Ly6C	Lymphocyte antigen 6 family member C
Macs	Macrophages
MCP1	Monocyte chemoattractant protein
MDP	Macrophage and dendritic cell progenitor
MG	Mammary gland
MHC	Major histocompatibility complex
Ms4a3	Membrane-spanning 4-domains, subfamily A, member 3
Myf5	Myogenic factor 5
NFD	Normal fat diet
Nur77	Nuclear receptor subfamily 4 group A member 1 – NR4A1
P7	Postnatal day 7
PAMPs	Pathogen-associated molecular patterns
Pax	Paired box
PDGFcc	Platelet-derived growth factor cc
PGC1α	Peroxisome proliferator-activated receptor-gamma coactivator alpha
Plvap	Plasmalemma vesicle associated protein
PPAR	Peroxisome proliferator-activated receptor
PRDM16	PR domain zinc-finger protein 16
PRRs	Pattern recognition receptors
SAMs	Sympathetic neuro-associated macrophages
scRNAseq	Single-cell RNA sequencing
Slc6a2	Solute carrier family 6 member 2
SNS	Sympathetic nervous system
SVF	Stromal vascular fraction
T2D	Type 2 diabetes
TAMs	Tumor associated macrophages
tdTomato	Tandem dimer tomato
TGFβ	Transforming growth factor beta
TH	Tyrosine hydroxylase

TIM4	T Cell Immunoglobulin And Mucin Domain Containing 4
TLR	Toll-like receptor
TNFα	Tumor necrosis factor alfa
TREM2	Triggering receptor expressed on myeloid cells 2
UCP1	Uncoupling protein 1
UMAP	Uniform Manifold Approximation and Projection
VAMs	Vascular associated macrophages
VEGFA	Vascular endothelial growth factor A
WAT	White adipose tissue
WHO	World health organization
Wnt	Wingless-related integration site
Wt1	Wilm's tumor gene

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 Norma Jäppinen, Inês Félix, Emmi Lokka, Sofia Tyystjärvi, Anne Pynttäri, Tiina Lahtela, Heidi Gerke, Kati Elima, Pia Rantakari, Marko Salmi. Fetal-derived macrophages dominate in adult mammary glands. *Nature communications*, 2019; 10, 281.

II Inês Félix, Heli Jokela, Joonas Karhula, Noora Kotaja, Eriika Savontaus, Marko Salmi, Pia Rantakari. Single-Cell Proteomics Reveals the Defined Heterogeneity of Resident Macrophages in White Adipose Tissue. *Frontiers in immunology*, 2021; 12, 719979.

III Inês Félix, Janni Ollikainen, Afra Haque, Joonas Karhula, Lauriina Tola, Marko Salmi, Heli Jokela, Pia Rantakari. Embryonic macrophages in brown adipose tissue interact with sympathetic neurons and correlate with the regulation of early-life thermogenesis. *Manuscript*.

1 Introduction

Adipose tissue, also known as fat, is a ubiquitous connective tissue distributed throughout the body. It can be found under the skin (subcutaneous fat) as well as between internal organs (visceral fat). In the case of the mammary gland (MG), the size of the fat pad directly determines the volume of the breast. There are three types of adipose tissue, white (WAT), brown (BAT) and beige. This thesis focuses on the study of MG, epididymal (e)WAT and interscapular (i)BAT resident macrophages **Figure 1**.

Macrophages have a central role in the immune system, and they are distributed in various tissues and organs throughout the body, including adipose tissue. Macrophages are traditionally associated with the process of inflammation, and are frequently seen as harmful cells. Nevertheless, macrophages are also known to be essential to the homeostasis, development and function of several tissues, being key cells for the healthy development of the organism (Nobs and Kopf 2021; Smith et al. 2007). Moreover, macrophages have the capacity to populate various tissues at different stages of embryonic development, originating from either the yolk sac or fetal liver, and during postnatal growth, deriving from the bone marrow. Currently, it is thought that the origin of tissue resident macrophages influences their functionality within the tissue they seed. Thus, macrophages are potential targets for developing therapeutic strategies for different diseases, like cancer or obesity.

The MG is based on a WAT fat pad, the mammary fat pad. The interaction between macrophages and adipocytes appears to be essential in different stages of MG development. As this organ is extremely plastic, it is predisposed to the development of neoplasia. Notably, the incidence of breast cancer continues to increase worldwide (Sung et al. 2021), meaning that the current therapies are not enough to control the development of this disease. In this study, we examined the origin and function of the different MG macrophages throughout development. The majority of the macrophages found in steady state adult MG originate from the fetal liver, and already populate the tissue at embryonic day E16.5, having a crucial role in the development and function of the MG.

Additionally, obesity is escalating worldwide and is associated with several health problems, like type 2 diabetes and cancer (Scully et al. 2021). Excessive

energy storage associated with obesity is accumulated in WAT. The pathophysiology of obesity has been widely studied, and macrophages were connected to an aggravation of the symptoms (Cai, Huang, and He 2022). Here I present a unified approach to the resident macrophage subtyping, and describe that WAT resident macrophages have a conserved function within the tissue, and are not responsive to diet changes. Moreover, I found that macrophages in WAT can have both embryonic and bone marrow origins.

As BAT has a fat-burning capacity, it is an interesting target for treating obesity (Poher et al. 2015). Macrophages in BAT have been described as crucial to the functionality of the tissue (Gallerand et al. 2021; Pirzgalska et al. 2017; Wolf et al. 2017). However, the current literature has only studied BAT macrophages in the mature tissue. Here I describe that the fetal liver-derived macrophages are the main population found in BAT during embryonic development and the perinatal phase.

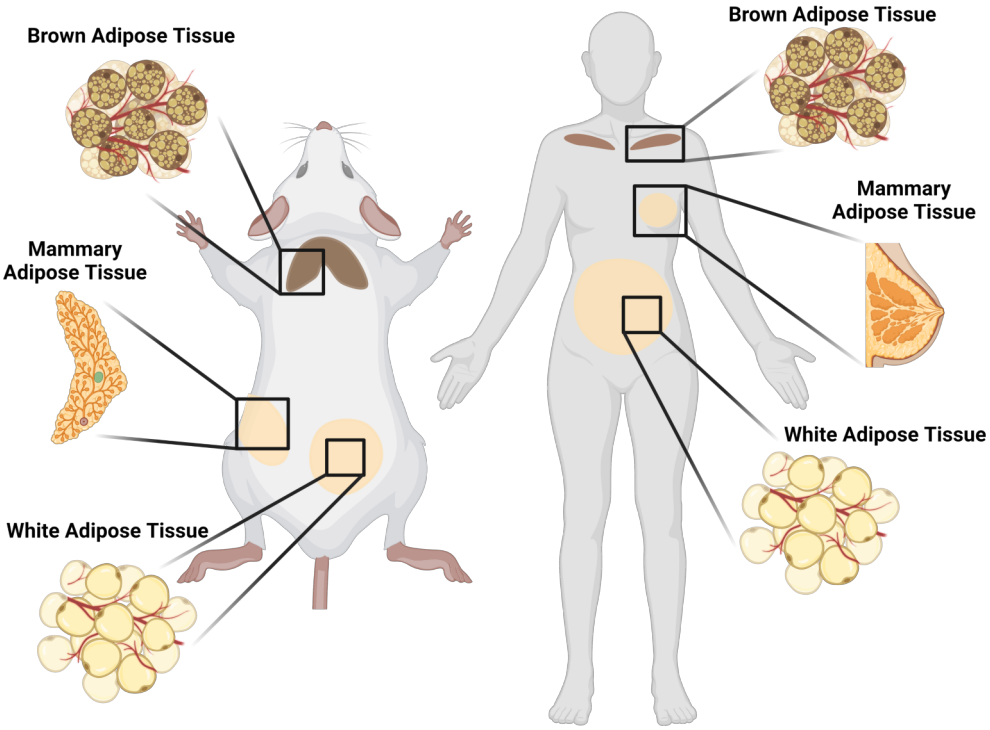

Figure 1 Representative distribution of the fat pads of interest in mouse and human. This project focuses on adipose tissues in the murine model, specifically: 4th mammary gland (I); epididymal white adipose tissue (II); interscapular brown adipose tissue (III). Created with BioRender.com

2 Review of the Literature

2.1 The immune system

Life forms have evolved from unicellular to multicellular organisms, with different cells having specific functions but working together to maintain life. Animals are complex multicellular organisms with organs, tissues, and cells composing the different functional systems that support the survival of the organism. The immune system is one of the essential systems, as it confers an evolutionary advantage to the organism to fight threats – the more complex the host organism, the more diverse and intricate the immune response. The immune system not only recognizes and destroys pathogens that are a threat to the animal but also allows for the survival of an identified non-self-organism if it is beneficial to the host. Importantly, the immune response can be triggered by foreign structures (such as, epitopes or pathogen-associated molecular patterns - PAMPs), as well as by injured or tumorous self-cells. This trigger can initiate a local immune response as well as a systemic one, as immune cells have the ability to circulate throughout the body, which enables them to reach and respond to injury in virtually any location. Moreover, the immune response activates two different subsystems that are deeply interconnected, the innate and the adaptive immune systems. The innate immune system will almost immediately find the pathogen and eliminate it in an antigen non-specific manner. Innate immune cells can also present epitopes from the pathogen to the adaptive immune cells, initiating a more specific and efficient immune response. Selected adaptive immune cells can generate immunological memory, which decreases the time needed to initiate an adaptive immune response in case of a reoccurring infection (Marshall et al. 2018).

2.1.1 Innate immune system

The innate immune system is the first line of defense of the organism, and it is constituted by (1) a physical barrier – that protects against pathogens or physical threats; (2) a chemical barrier – that inhibits pathogen growth, shields against invasion, and destroys pathogens; and (3) immune cells – that recognize pathogens, induce an immune response and recruit more cells to the site of infection (Gallo and Nizet 2008). Epithelial cells constitute the primary barrier against pathogens. Apart from skin,

several mucosal epithelial surfaces exist within the body – urogenital, digestive, respiratory, and ocular. Mucosa produces not only mucins that act as a physical barrier but also chemicals such as antimicrobial peptides that destroy pathogens and inhibit pathogen-host cell interaction (Liévin-Le Moal and Servin 2006). Similarly, the complement system acts as a chemical barrier, in this case in blood circulation, tissues, cell surface, or intracellularly. The complement system is composed of several soluble proteins that act in a cascade of events that culminate in pathogen lysis. The complement system also opsonizes pathogens signaling to the immune cells to activate their response, amplifying and modulating innate and adaptive immune systems (Merle et al. 2015). Thus, the innate recognition system for pathogens or injured cells is triggered not only by the innate immune cells but also by the previously mentioned constituents of the innate immune system. This mechanism is essential for triggering the immune response, and depends on pattern recognition receptors (PRRs). These receptors distinguish healthy self-molecules from PAMPs, such as LPS (lipopolysaccharides), as well as endogenous molecules released by cells in response to stress or damage, known as damage/danger-associated molecular patterns (DAMPs), such as extracellular HSP (heat shock proteins) (Amarante-Mendes et al. 2018). If a pathogen evades the physical and chemical barrier, it can still be detected by the PRRs expressed not only by the patrolling immune cells, but also by the epithelial cells that constitute the primary barriers. This initiates the immune response, leading immune cells to migrate toward the infection/injury site.

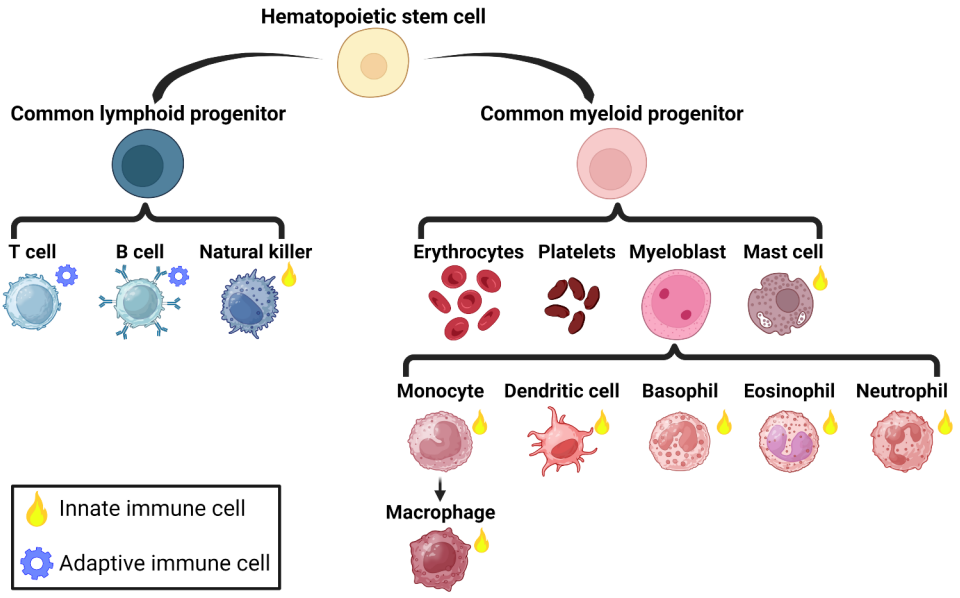

Figure 2 Hematopoiesis. Simplified tree of the development of the major lineages of blood cells. Created with BioRender.com

2.1.1.1 Hematopoiesis

All blood cells, including immune cells, are formed in a process called hematopoiesis. The hematopoietic stem cell (HSC) is recognized as the mother of all the cells from blood lineage, it has a multipotent profile, and it is able to self-renew at a high rate. From the HSC, the lineage can be separated into myeloid or lymphoid. The common lymphoid progenitor (CLP) is responsible for producing lymphocytes T and B, as well as natural killer cells. In contrast, the common myeloid progenitor (CMP) generates thrombocytes/platelets, erythrocytes, mast cells, and myeloblasts. Myeloblasts further differentiate into basophils, eosinophils, neutrophils, dendritic cells, and monocytes, which in turn, give rise to macrophages (Galloway and Zon 2003; Laurenti and Göttgens 2018) **Figure 2**.

The HSC is identified in murine embryonic development around embryonic day (E)10.5, but circulating erythrocytes, macrophages, and platelets settle as early as E7.5 (McGrath and Palis 2005). Thus, embryonic hematopoiesis is dependent on different progenitor cells prior to the establishment of HSCs. Studies in mice have identified distinct waves of hematopoiesis, characterized by differences in complexity, progenitor cell types, and the hematopoietic organs involved. Primitive hematopoiesis takes place in the blood islands of the extraembryonic yolk sac, around E7.5 to E9.5. The primitive blood cells are primarily nucleated erythrocytes and macrophages (McGrath and Palis 2005; Palis 2016). In parallel, transient definitive hematopoiesis initiates in the yolk sac's hemogenic endothelium around E8, with the surge of erythromyeloid progenitors (EMPs) and lymphoid precursors (Palis and Yoder 2001; Perdiguero and Geissmann 2015). EMPs are multipotent progenitor cells that differentiate into the erythroid or myeloid lineage, having a more restricted differentiation potential when compared to HSCs, and mainly produce immature erythrocytes and macrophages (McGrath et al. 2015). EMPs circulate in the embryo and transiently seed the fetal liver around E9, where they expand and reach their differentiation potential until around E16.5 (Perdiguero et al. 2015). Definitive hematopoiesis overlaps with previous hematopoietic waves and extends from late fetal development throughout postnatal life (Ivanovs et al. 2014; Ng and Alexander 2017; Palis 2016). It begins with the influx of HSCs originating from the hemogenic endothelium of the aorta-gonad-mesonephros region into the fetal liver, around E10.5 (Ema and Nakauchi 2000). The fetal liver's niche provides HSCs nutrition, fomenting their expansion and regulating their functionality (Lewis, Yoshimoto, and Takebe 2021). Accordingly, the fetal liver is the primary site for embryonic definitive hematopoiesis. HSCs from the fetal liver enter the circulation and begin to populate the spleen around E15.5, followed by colonization of the bone marrow around E16.5, which coincides with the establishment of vascularization and ossification. Hematopoiesis in the spleen is transient, occurring mainly around the perinatal period. In the bone marrow, HSCs proliferate during the neonatal period

and later become quiescent. Bone marrow is the major hematopoietic organ during postnatal life, offering the microenvironment for the maintenance of HSCs and regulating the differentiation process (Gao et al. 2018).

2.1.2 Macrophages

Macrophages, from the Greek "*makro phagein*" meaning "big eater", have the primary function of phagocytosing and destroying pathogens or damaged cells. Macrophages exist in all tissues of the body and play a pivotal role in the maintenance of tissue homeostasis (Hirayama, Iida, and Nakase 2018).

2.1.2.1 Function

Macrophages are characterized as phagocytes, like dendritic cells and neutrophils. Thus, their main function has been associated to their capacity of engulfing and digesting pathogens or injured cells. The process of phagocytosis starts with the detection of the foreign pathogen or injured cell, by the PRR. There are different families of PRRs, some induce phagocytosis upon PAMP or DAMP detection, while others prepare the macrophage for phagocytosis without directly inducing it (Uribe-Querol and Rosales 2020). In the context of phagocytosis-inducing receptors, macrophages express several distinct phagocytic receptors, classified in accordance with the specific types of ligands they recognize (Hirayama et al. 2018). For example, CD163 (cluster of differentiation 163) and CD206 (mannose receptor, coded by *Mrc1* gene) are two scavenger receptors highly expressed by macrophages. CD163 is also known as hemoglobin/haptoglobin scavenger receptor and it plays a pivotal role on the removal of hemoglobin/haptoglobin complexes from circulation, also promoting erythroblast growth and survival. CD206 is essential for the clearance of several molecules as it binds to different kinds of ligands, being described as a *bona fide* phagocytic receptor. Interestingly, CD206 is also involved in the process of antigen presentation (Nielsen et al. 2020). Once the particle binds to the receptors the process of phagocytosis starts as the cell membrane covers the particle until it surrounds it and closes, creating a new intracellular macrophage component, the phagosome. The phagosome merges with lysosomes and the pathogens are digested by the action of different enzymes (Uribe-Querol and Rosales 2020).

Macrophages can also activate the adaptive immune system by acting as antigen-presenting cells (APCs). Antigen cross-presentation is achieved when peptides from the degraded pathogen proteins are processed via the major histocompatibility complex (MHC) class I or II present on their cell surface, serving as effectors for cell-mediated immunity (Cho et al. 2014; Morris et al. 2013). During the antigen

presentation, T cell activation and survival is also dependent on costimulatory molecules expressed on the cell surface of the macrophage, which interact with the T cell receptor. Additionally, coinhibitory molecules are also found in macrophages, which downregulate T cell activation. Furthermore, the existing cytokines produced by macrophages and other cells also influence the success of the antigen presentation, such as interleukin 12 (IL-12) or IL-6 (Guerriero 2019).

As well as their immune functions, macrophages also exhibit important roles during the development of the tissues, the maintenance of homeostasis and in repair and regeneration (Mosser, Hamidzadeh, and Goncalves 2020). During development, for example, macrophages are crucial for the remodeling of the bone, establishing the cavities where bone marrow settles and starts its hematopoietic function (Pollard 2009). Moreover, macrophages clear the tissues from injured, senescent or dying cells, being crucial for tissue patterning, growth, and renewal. For example, macrophages support erythropoiesis by engulfing the nuclei from the mature erythroblasts originating the erythrocytes (Wynn, Chawla, and Pollard 2013). In addition, macrophages play a crucial role in the branching of the mammary gland, not only by clearing the developing tissue from apoptotic cells, but also by interacting with the mammary stem cells, regulating the tissue remodeling (Gyorki et al. 2009).

Macrophages interact with their surroundings by being sensitive to other cells' cytokines, and also by producing several cytokines themselves. These cytokines can be associated with the inflammatory process and the recruitment of leukocytes to sites of infection, as well as to the homeostasis and repair of the tissues, (Fujiwara and Kobayashi 2005; Mosser et al. 2020). Macrophages are able to rapidly increase or decrease the transcription of these cytokines according to the appropriate stimuli, as well as their degree of maturation and the surrounding microenvironment (Cavaillon 1994).

Currently, macrophages are no longer perceived solely as having an immune function, as they play a pivotal role in various physiological processes linked to tissue health and homeostasis (Park et al. 2022). Macrophages have several functions and are very plastic cells, rapidly shifting their phenotypes in response to cell interactions, cytokines, hormones, and several other chemical signals (Das et al. 2015; Ovchinnikov 2008). The diversity of macrophage phenotypes and functions has prompted extensive research that aimed the classification of the various types of macrophages.

2.1.2.2 Macrophage classification in vitro

The tissue environment shapes the gene expression of macrophages, promoting the specialization of different subsets within the same tissue, and from tissue to tissue

(Gosselin et al. 2014). The characterization of macrophage phenotypes has previously been based on the different polarizations exhibited *in vitro*, where the dichotomy between pro- and anti-inflammatory cytokine release and response is more obvious, see **Figure 3A**. Briefly, classically activated or M1 macrophages, are stimulated by toll-like receptor ligands (e.g., LPS) and IFNγ (interferon gamma, produced by activated T cells and NKs), and produce high levels of pro-inflammatory cytokines, like TNF (tumor necrosis factor – previously TNFα), IL-1β or IL-6. Whereas the alternatively activated or M2 macrophages are activated by IL-4 or IL-13 and produce anti-inflammatory cytokines and growth factors, like IL-10 and TGFβ (transforming growth factor beta). M1 macrophages are crucial for the inflammatory process, promoting leukocyte chemotaxis at the site of infection, and have strong microbicidal and tumoricidal activity. M2 macrophages are essential in the resolution phase of inflammation by helping clearing debris, as they have high expression of scavenging molecules such as CD163 and CD206. This scavenger function promotes tissue regeneration, and restores tissue homeostasis (Mills et al. 2000; Sica and Mantovani 2012; Sindrilaru et al. 2011).

However, this division between M1 and M2 macrophage polarization is artificial and is not a reliable categorization for *in vivo* purposes. According to dynamic tissue-environment signals, macrophages can constantly change their phenotypes (Okabe and Medzhitov 2014), see **Figure 3B,** which emphasizes the importance of an improved classification system.

2.1.2.3 Macrophages classification systems

The macrophage landscape within a tissue is highly dynamic and does not conform to the conventional M1/M2 paradigm. Other approaches to the macrophage profiling/classification have been proposed. For example, as the surrounding environment has a significant influence on macrophage function it is possible to describe macrophages according to their location within the tissue and link it to their function, such as vascular associated macrophages (VAMs) in a steady state adipose tissue. These macrophages are located close to the vessels and actively survey the tissue by scavenging macromolecules, readily responding to environmental changes (Silva et al. 2019).

Moreover, macrophages adopt specific functions according to the tissue they reside in, hence the name “tissue resident macrophages”. A noteworthy example are microglia, specialized macrophages residing in the brain. Microglia engage with neuronal cells not only by phagocytosing dying neurons or neuronal branches through a process known as pruning, but also by controlling blood flow in the brain parenchyma through the production of anti-inflammatory and angiogenic cytokines (Császár et al. 2022). Beyond adopting an M2-like phenotype, microglia can also

manifest a pro-inflammatory profile, resembling an M1-like phenotype, which has been implicated in the exacerbation of conditions such as Alzheimer's and Parkinson's diseases (Lucin and Wyss-Coray 2009). This example supports the notion that macrophages are very plastic cells, and that the surrounding microenvironment is crucial for the phenotypic polarization of these cells (Park et al. 2022), **Figure 3**. Interestingly, microglia originate from yolk sac and play a pivotal role in the brain's early developmental stages, contributing significantly to its overall well-being (Ginhoux et al. 2010). Overall, tissue resident macrophages can have three different precursors: yolk sac, fetal liver, or bone marrow (Dick et al. 2022). It is known that the timing of macrophage seeding in tissues during development may impact their functionality, as well as influence tissue function and development (Park et al. 2022). Thus, it is imperative to consider that the developmental origin of macrophages and the timing of their migration to the tissues can also significantly impact their functionality and phenotype (Park et al. 2022).

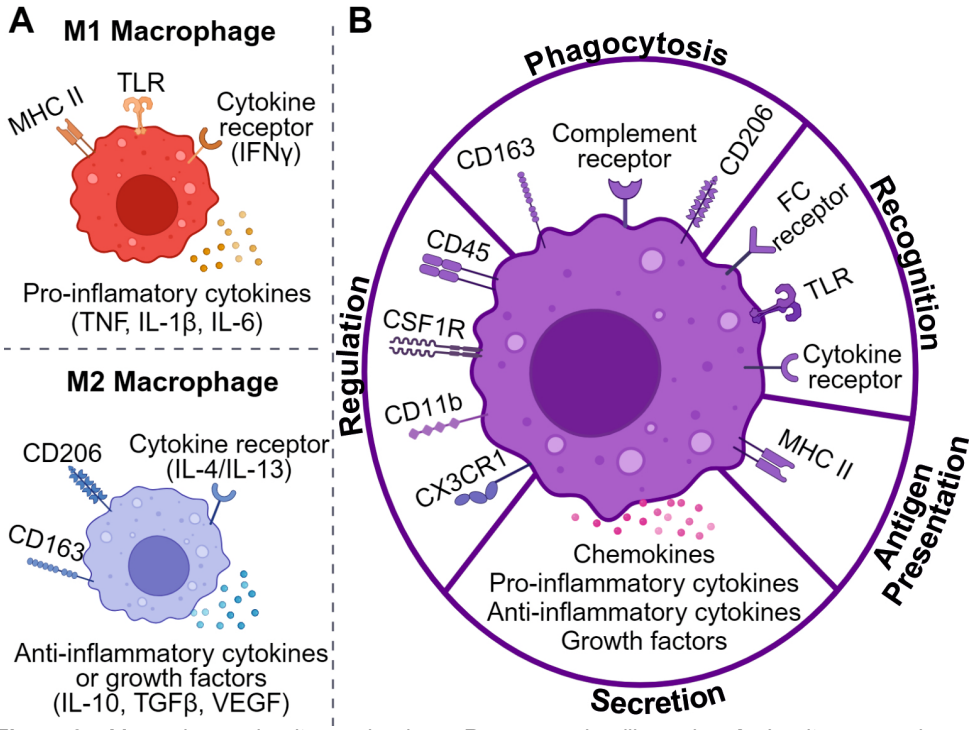

Figure 3 Macrophages *in vitro* vs *in vivo* – Representative illustration **A.** *In vitro* macrophages behave either as M1 – pro-inflammatory or M2 – anti-inflammatory, expressing a specific set of cell markers, in response to certain stimuli. **B.** *In vivo* macrophages are equipped with multiple receptors that facilitate an appropriate response to the microenvironmental stimuli. Macrophages exhibit a dynamic phenotype that adapts along a flexible spectrum, instead of having a distinct set of inflammatory states. Adapted from *"Macrophage Polarization: M1 and M2 Subtypes", by BioRender.com (2023). Retrieved from* https://app.biorender.com/biorender-templates and *(Lucin and Wyss-Coray 2009) – Figure 1.*

2.1.3 Macrophage ontogeny

Tissue resident macrophages are long-lived cells with the remarkable ability to self-renew within tissues without relying on circulating precursors. Thus, macrophages originating from the first hematopoietic wave can persist and maintain themselves in the tissues they populate throughout postnatal life, see **Figure 4**. There are three hematopoietic waves: the primitive, the transient definitive and the definitive wave. The first two waves happen during embryonic development and the last one starts around birth. As mouse studies have paved the way to understand macrophage origin and kinetics, particularly, with the use of fate mapping mouse models, the three waves will be described from a murine perspective.

2.1.3.1 Fetal-derived macrophages

In mice, during early embryonic development in the extraembryonic yolk sac blood islands, the first macrophages are formed between E7 and E9.5 in a CSF1 (colony stimulating factor 1) -dependent manner and without a monocytic progenitor. With the establishment of a circulatory system at E8.5, these macrophages enter into circulation, seed the tissues and mature. Primitive macrophages populate the brain as early as E9, and the fetal liver around E10.5 (Ginhoux et al. 2010; Palis 2016). Interestingly, this first hematopoietic wave is short-term and by E9.5 no primitive erythroid progenitors are detected. Concurrently, yolk sac hemogenic endothelium provides the origin for the first EMPs that can differentiate already into macrophage progenitors around E8.5. Yolk sac EMP-derived macrophages enter into circulation and seed several tissues, see **Figure 4**. Meanwhile, EMPs also circulate and seed the fetal liver around E10.5. The fetal liver niche provides the optimal signaling environment for EMPs to achieve their differentiation potential. Consequently, the first monocytes begin to appear in the bloodstream at E12.5, differentiating into macrophages when migrating into the tissues (McGrath et al. 2015). Notably, fetal liver monocytopoiesis is the main source of macrophages that populate most tissues. These macrophages are also long lived and self-maintaining throughout postnatal life, and in most tissues the yolk sac-derived macrophages are partially substituted by E16.5. Another source of fetal liver hematopoiesis are HSCs from the aorta-gonad-mesonephros (AGM) that seed the fetal liver at E10.5 and start producing monocytes soon after, see **Figure 4**. This last wave of hematopoietic progenitors is considered definitive, as all blood cells originate from HSCs (Ng et al. 2023). Finally, around E16 the HSCs migrate from the fetal liver to the bone marrow, and become functional progenitor cells around day E17.5 (Clapp et al. 1995; Lewis et al. 2021; Mikkola and Orkin 2006). The HSC pool expands within the bone marrow, and even though the liver maintains its hematopoietic capacity for around two weeks after birth, the bone marrow rapidly becomes the main hematopoietic organ

throughout postnatal life (Christensen et al. 2004; Shevyrev et al. 2023; Wright et al. 2001).

2.1.3.2 Bone marrow-derived monocytes

After birth, the HSCs populate and self-renew in bone marrow during postnatal life, see **Figure 4**. To give origin to a monocyte, a Myb (transcription factor involved in cell cycle regulation, DNA replication, and maintenance of genomic integrity) - dependent HSC (Soza-Ried et al. 2010) has to divide and differentiate in different hematopoietic progenitor cells, losing its self-renewal capacity while committing to the mononuclear phagocyte lineage. The known intermediate of monocyte progenitors are the CMP, which provide the origin of erythrocytes and megakaryocytes, and granulocyte and macrophage progenitors (GMP). GMPs differentiate into macrophage and dendritic cell progenitors (MDP), but can also commit to the neutrophil fate. MDPs are precursors to dendritic cells and to the common monocyte progenitor (CMoPs), that then differentiate into pro-monocytes and subsequently into monocytes that enter into the circulation (Pittet, Nahrendorf, and Swirski 2014)

Differentiation into monocytes, from the common myeloid progenitor is also driven by the CSF1 cytokine, and these cells are characterized by the expression of CSF1R (macrophage colony stimulating factor 1 receptor - also known as CD115) and CD117 (also known as c-Kit) (Hettinger et al. 2013). Once monocytes enter into circulation, they are known to express Ly6C (lymphocyte antigen 6 family member C) surface membrane protein. Two subsets of circulating monocytes have been described: one expressing high levels of Ly6C ($Ly6C^{High}$) and CCR2 (C-C chemokine receptor type 2) as this subset is dependent on CCL2 (C-C chemokine ligand type 2) chemoattractant for migration and tissue entry, and low levels of CX3CR1 (C-X3-C motif chemokine receptor 1) (Boring et al. 1997; Davies et al. 2013; Lu et al. 1998; Serbina and Pamer 2006; Tsou et al. 2007); and another subset expressing low levels of Ly6C ($Ly6C^{Low}$), but high levels of CX3CR1, being mainly dependent on Nur77 transcription factor (nuclear receptor subfamily 4 group A member 1 – NR4A1) (Hanna et al. 2011; Martínez-González and Badimon 2005; Mullican et al. 2007). The $Ly6C^{High}$ subset is recruited to inflammation sites, whereas the $Ly6C^{Low}$ subset remains in the circulation as vessel patrolling cells, not normally extravasating to the inflamed tissues (Hanna et al. 2011). The $Ly6C^{High}$ $CCR2^{+}$ monocyte subset is called into action during inflammation (Xu et al. 2005), and once these monocytes perform diapedesis in the tissue, they differentiate into macrophages in the presence of CSF1 (Yang et al. 2014), or into monocyte-derived dendritic cells (Segura and Amigorena 2013). Interestingly, $Ly6C^{High}$ $CCR2^{+}$ monocytes can also enter the tissues in a steady

state without differentiating, and keeping their monocytic signature while patrolling the tissue (Jakubzick et al. 2013).

Bone marrow-derived macrophages are able to stay within the tissue and resemble tissue resident macrophages, and are also able to reconstituting the resident pool of macrophages. However, not all subsets of tissue resident macrophages are able to be replenished by bone-marrow, as is the case of testicular and alveolar (van de Laar et al. 2016) macrophages.

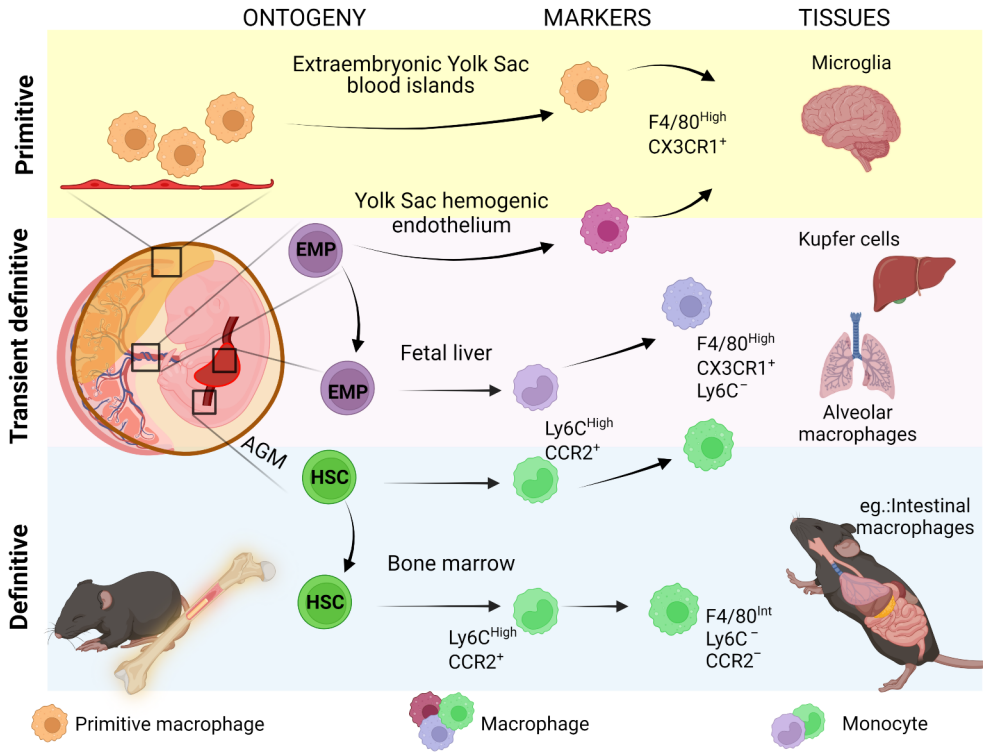

Figure 4. Ontogeny of tissue resident macrophages throughout development. Macrophages arise to the tissues from 3 different waves of hematopoiesis. A first wave of primitive macrophages from extraembryonic yolk sac blood islands will start populating tissues without a monocyte intermediate. The second wave is erythromyeloid progenitor (EMP) –dependent. It initiates in yolk sac hemogenic endothelium, still without a monocyte progenitor. The EMPs then migrate to fetal liver where the differentiation potential is achieved and monocytes start being produced. The monocytes infiltrate the tissues and differentiate to macrophages. The last wave is hematopoietic stem cell (HSC) –dependent, it starts with HSCs arising to fetal liver from the aorta-gonad-mesonephro (AGM) region. Before birth, HSCs migrate from the fetal liver to the bone marrow, where they mature and start producing monocytes. Created with BioRender.com

2.1.4 Tissue resident macrophages

Each adult tissue has its own particular composition of coexisting embryonic and adult bone marrow -derived macrophages (Dick et al. 2022). These macrophages are commonly called tissue resident macrophages, they self-maintain by proliferating locally, and are sensitive to different cell stimuli, such as cytokines, being specialized according to the tissue environment (Lavin et al. 2014).

Brain macrophages, microglia, settle in the tissue during the primitive wave of hematopoiesis. Microglia are self-maintaining throughout postnatal life, and as the blood-brain barrier forms, there is limitation to new macrophage waves. Only in the context of injury will new monocyte-derived macrophages substitute microglia **Figure 4** (Ginhoux et al. 2010; Ginhoux and Guilliams 2016; Hoeffel et al. 2012, 2015; Hoeffel and Ginhoux 2015; McGrath et al. 2015; Squarzoni et al. 2014), see. Importantly, during development, microglia support specific niche functions such as synaptic pruning or angiogenesis (Fantin et al. 2010; Paolicelli et al. 2011).

Macrophages derived from fetal liver are mainly EMP-dependent and have a monocytic precursor. This is the case, for example, of Kupfer cells in the liver (Hoeffel et al. 2015; Perdiguero et al. 2015; Schulz et al. 2012; Sheng, Ruedl, and Karjalainen 2015), Langerhans cells in the skin (Ginhoux and Merad 2010; Kaplan 2017; Romani, Schuler, and Fritsch 1986) or alveolar macrophages in the lung (Evren, Ringqvist, and Willinger 2020; Guilliams et al. 2013), see **Figure 4**. Our group has also described a fetal liver origin for specific subsets of macrophages in MG (Jäppinen et al. 2019), testis (Lokka et al. 2020) and ovary (Jokela et al. 2020).

A general distinction between the two macrophage subsets described during embryonic development, is done according to the differential expression of the F4/80 (coded by adhesion G protein-coupled receptor E1 gene – *ADGRE1*) membrane cell marker. In summary, during embryonic development F4/80 is highly expressed by yolk sac-derived macrophages, and fetal liver-derived macrophages have an intermediate to low F4/80 expression. After birth, when bone marrow-derived macrophages start to populate the tissues expressing intermediate levels of F4/80, fetal liver macrophages increase their F4/80 expression, resembling the yolk sac-derived subset, indicating a mixed origin of F4/80^{High} macrophages in adults (Ginhoux and Guilliams 2016; Hashimoto et al. 2013; Perdiguero and Geissmann 2015; Schulz et al. 2012; Varol, Mildner, and Jung 2015; Yona et al. 2013), see **Figure 4**.

2.1.5 Mouse models to study macrophage ontogeny

Tissue resident macrophages are present in all body tissues and can originate from three distinct waves of hematopoiesis, as illustrated in **Figure 4**. Therefore, employing precise tools that target these diverse macrophage lineages is essential for investigating their respective functions in each tissue.

2.1.5.1 Fate mapping mouse models

Mouse models that allow the labelling of a certain cell type during a specific time of development have assisted immunologists to unravel the developmental origins of different immune cells, including macrophages. These mice are usually Cre-ER (cyclization recombination - estrogen receptor) crossed with reporter LoxP (locus of crossing [x-ing]-over of bacteriophage P1) mice.

Cre is an enzyme that facilitates genetic recombination by catalyzing the rearrangement of DNA sequences known as LoxP. This process leads to the removal of the DNA segments located between two LoxP sites. Typically, Cre recombinase is positioned downstream of a specific gene's promoter. Consequently, Cre-expressing cells are those that produce the protein of interest. To target specific lineage cells, the Cre recombinase must be linked to the promoter of a gene that is selectively active in cells of the desired lineage (Lee, Rudd, and Smith 2022).

Reporter mice have LoxP sites located downstream of a ubiquitous promoter (active in all cell types, such as Rosa26). These LoxP sites flank a stop sequence, preventing the expression of a fluorescent protein gene, such as EYFP (enhanced yellow fluorescent protein) or tdTomato (tandem dimer tomato). When a Cre mouse is bred with a reporter mouse, the Cre recombinase acts on the LoxP sites, removing the stop sequence. This, in turn, enables the expression of the fluorescent protein in cells that produce the protein under investigation (Lee, Rudd, and Smith 2022).

Additionally, an inducible Cre system has been developed to label specific lineage cells at precise developmental time points. The Cre-ER recombinases are ligand-dependent enzymes, remaining inactive until they are induced by tamoxifen, a synthetic estrogen receptor ligand (Feil, Valtcheva, and Feil 2009).

In this project, we used four different tamoxifen-inducible models, with the Cre enzyme associated with *Csf1r*, *Cx3cr1*, *Ccr2*, or *Ms4a3* genes. The *Csf1r* gene is widely expressed in macrophages, as the differentiation and maturation of macrophages is dependent on CSF1, which is the ligand for the CSF1r. Moreover, macrophage migration, proliferation, and survival is also regulated by CSF1 (Auffray et al. 2009; Hume and MacDonald 2012). Interestingly, *Csf1r* is expressed in the early macrophage progenitors from the primitive hematopoietic wave, thus, this reporter model is used to study yolk sac-derived macrophages (Ginhoux et al. 2010; Hoeffel et al. 2015; Perdiguero et al. 2015). The *Cx3cr1* gene codes the fractalkine chemokine receptor – CX3CR1, which is widely expressed in monocytes and macrophages (Jung et al. 2000), and also known to be expressed in yolk sac-derived macrophages (Yona et al. 2013). The *Ms4a3* gene codes the membrane-spanning 4-domains, subfamily A, member 3 (Ms4a3) protein; expressed in GMPs, and this reporter is used to label bone marrow-derived macrophages (Liu et al. 2019). Lastly, the *Ccr2* gene is expressed in monocytes and macrophages, originating from

fetal liver or bone marrow (Croxford et al. 2015; Dick et al. 2022; Hoeffel et al. 2015).

Accordingly, the Cre-LoxP system has important applications in the study of cell lineages and cell tracing. However, this system can have limitations such as: the variation of the labelling efficiency; the possibility of Cre cellular toxicity; Cre leakage (background Cre activity without tamoxifen induction); deficient target gene expression; the specificity of the gene of interest (most genes are not exclusively expressed only by one cell type); side or off-target effects of tamoxifen; or the discrepancy of the tamoxifen effects according to the administration route (Becher, Waisman, and Lu 2018; Ilchuk et al. 2022). Therefore, it is essential to consider these limitations when interpreting the data generated by mouse models and to employ appropriate controls in experimental design.

2.1.5.2 Macrophage deficient mice

Genetically modified mice with deletions in genes crucial for monocyte or macrophage function, migration, maturation, or survival are used to elucidate the roles of specific macrophage subsets. This approach helps uncover the functions associated with these subsets by evaluating the abnormalities in the mutant mice compared to tissue homeostasis in WT mice. For example, because of their specificity and strict influence in the monocyte subsets, either for production, migration or tissue infiltration, $Ccr2^{-/-}$or $Nur77^{-/-}$ mouse models are often used to understand the function of the different monocytic subsets in the context of disease, or to study the contribution of these bone marrow-derived subsets to the tissue macrophage pool (Boring et al. 1997; Hanna et al. 2011; Serbina and Pamer 2006). Furthermore, the osteopetrotic mice ($Csf1^{op}$), a CSF1-deficient mouse model, have a systemic depletion of several macrophage subsets, particularly in osteoclasts, leading to the development osteopetrosis (Hua et al. 2018).

Interestingly, $Plvap^{-/-}$ mice show a diminished frequency of tissue resident macrophages in several tissues, however the plasmalemma vesicle associated protein (PLVAP) is a protein specific to the endothelial cells (Rantakari et al. 2016). PLVAP is mostly associated to the blood vessel endothelium, but is also expressed in lymphatic endothelial cells (Rantakari et al. 2015). This protein is involved in the formation of the diaphragms that connect the endothelial fenestrae, and it is also required for the formation of the stomata in endothelial caveolae (or plasmalemmal vesicles) (Denzer et al. 2023). Thus, it has a critical role the microvascular permeability, and leukocyte migration (Rantakari et al. 2015). PLVAP diaphragms are found in the fetal liver sinusoidal fenestrae since E12.5, but $Plvap^{-/-}$ mice lack these diaphragms during embryonic development. The migration of EMPs from the yolk sac to the fetal liver is normal in these mice, however, after maturation, the fetal

liver monocytes are not able to exit the liver and seed the tissues, leading to the diminished frequency of fetal liver macrophages in most tissues, and the accumulation of mature macrophages in fetal liver (Rantakari et al. 2016).

To examine the ontogeny and function of tissue resident macrophages it is essential to also understand developmental stages and the physiological function of the tissue under study. The three tissues of interest are mammary gland, white adipose tissue and brown adipose tissue.

2.2 Mammary gland (MG)

The MG, is one of the most distinctive evolutionary features of mammals. These glands are responsible for the production of milk that nourishes the offspring; they are well developed and undergo several functional changes during puberty, pregnancy and lactation in females and have a rudimentary development in males. The number of glands is species-specific, in the case of humans, one pair of glands located in the thoracic area. In contrast, mice have 5 pairs of glands, three in the thoracic and two in the ventral region. The first gland is the least developed and the 4th gland (inguinal) is the most developed. Overall, the MG has an epithelial structure constituted by a network of ducts, highly variable between individuals, terminating in alveoli, where milk is produced (Cristea and Polyak 2018). The ducts are lined with myoepithelial cells, which constitute a contractile epithelium that aids the expulsion of milk from the nipple during suction. Notably, the inner layer of the duct is constituted by luminal epithelial cells which differentiate into milk-producing alveolar epithelial cells (Gudjonsson et al. 2005). Murine MG stroma is based on a fat pad, being constituted by white adipocytes, epithelial cells, mesenchymal cells in particular fibroblasts, vascular endothelium, multiple lineages of stem cells and several immune cells. Interestingly, each fat pad has a draining lymph node (McNally and Stein 2017). The human MG is composed of lobes all draining into the nipple, and the branching of the lobes gives rise to lobules connected by ducts (Dontu and Ince 2015).

Breast cancer represents ¼ of all cancers diagnosed in women, and unfortunately the incidence rates are still rising, particularly in Northern and Eastern Europe (DeSantis et al. 2015). The murine model is one of the most common models to study breast cancer as mouse and human MG have several conserved genes and pathways (Lim et al. 2010).

2.2.1 MG development

The MG only fully develops after puberty being highly responsive to estrogens and progesterone, and undergoes several growth phases until mature. Once matured,

during pregnancy and lactation this gland responds to hormonal cues by developing its alveoli where the milk is produced, and involuting at the end of lactation (McNally and Stein 2017). To study MG function, it is important to understand the tissue's development and morphogenesis from the early embryonic stages to the fully functional state. This requires studying the cellular and molecular mechanisms that control the growth, branching, and differentiation of mammary tissue. I will focus on the developmental stages of murine MG, as the development of human MG has been described as somewhat similar to that of murine, being slightly more complex and divided by ten different phases (Russo and Russo 2004).

2.2.1.1 Prenatal development

The development of two milk lines initiates around E10, progressing to the formation of five pairs of ectodermal placodes. The placodes are thickened layers of epithelial tissue that subsequently invaginate into the dermal mesenchyme, forming the first MG buds at E12.5, and increase in size up to E15 (Cowin and Wysolmerski 2010), see **Figure 5**. In females, the mammary development is paused, but in males, the mesenchyme is sensitive to testosterone, and around E13.5-E15.5, it surrounds the bud leading to its degeneration (McNally and Stein 2017; Watson and Khaled 2008). In females, at ~E15.5 the buds elongate, by the proliferation of mesenchymal cells, from the tip, forming a sprout that invades the mesenchymal fat pad precursor. Each sprout grows on its inside, creating a hollow lumen that opens to form the nipple by epidermal invagination. Sprouts are already visible around E16 and their development is guided by fat pad precursor cell signaling, and at E18.5 the small glands that are present at birth are formed. At birth, there are 10-15 ductal branches but their growth is slowed until puberty (Couldrey et al. 2002; McNally and Stein 2017). Regulation of MG development is mainly achieved by mesenchyme and epithelium through canonical *Wnt* (wingless-related integration site) signaling, which acts at E10.5 in the formation of the mammary line, and also contributes to bud formation later in development. Many other factors contribute for the regulation of MG development, such as: the fibroblast growth factor family which guides the formation of the placodes; the parathyroid hormone related protein, expressed by the epithelial cells from the mammary bud during the formation of the hollow lumen, which is crucial for the mammary fate switch in mesenchymal cells, and for the nipple opening (Chu et al. 2004; Inman et al. 2015; McNally and Stein 2017; Veltmaat 2017).

2.2.1.2 Postnatal development

After birth, the MG is responsive to systemic hormones like estrogens and progesterone, which are regulated by the hypothalamus-pituitary-gonadal axis (Hennighausen and Robinson 2001; McNally and Martin 2011). Nonetheless, the mammary environment still plays a crucial role in regulating and guiding ductal network development (Fata, Werb, and Bissell 2004). The gland growth is somewhat quiescent until puberty and accompanies body growth (isomorphic) (Watson and Khaled 2008). At this point, the stroma of the gland is mostly constituted by fibroblasts and connective tissue, and the single ductal network is a rudimentary structure occupying only a fraction of the mammary fat pad. In humans, the MG at this stage is constituted of several small ductal networks that converge in the nipple (McNally and Stein 2017).

The onset of puberty, around P (postnatal day) 25 in female mice (Ismail, Garas, and Blaustein 2011), triggers the extensive proliferation of the terminal end buds, which are the main drivers of ductal morphogenesis. At this stage, there is a sudden growth not only of the gland, but also of the mammary stroma, comprised of the fat pad with fully differentiated white adipocytes and fibroblasts. The terminal end buds are club shaped structures at the end of the primary ducts that are highly mitotic, proliferating, elongating and bifurcating into new primary ducts that perform lateral branching invading further the fat pad, filling around 60% of the gland with ductal tree (Macias and Hinck 2012). The fat pad delineates the border of the branching expansion, and even though mice reach sexual maturity around 5 weeks of age, the MG continues its ductal branching until 8-12 weeks (McNally and Stein 2017; Watson and Khaled 2008). The MG reaches maturity when the extremity of the fat pad is reached and the terminal end buds shrink and stop proliferating. The murine MG ductal network is sensitive to the estrous cycle, fluctuating according to the hormonal cues from the cycle. Pubertal MG development is initiated by the production of estrogen by the ovaries, as the mammary epithelium, but not its stroma, is estrogen-dependent. Ductal expansion during puberty is maintained by both sexual and pituitary hormones, such as growth hormone, local growth factors, and cytokines (McNally and Martin 2011; McNally and Stein 2017), see **Figure 5**.

With every estrous cycle, there is the formation of lateral and alveolar buds subdividing into undeveloped alveolar structures. During pregnancy the MG goes through new ductal branch proliferation and alveolar bud formation, and one day before delivery, the alveoli mature, and become capable of secreting milk. These alveoli start involuting after weaning (McNally and Martin 2011), see **Figure 5**.

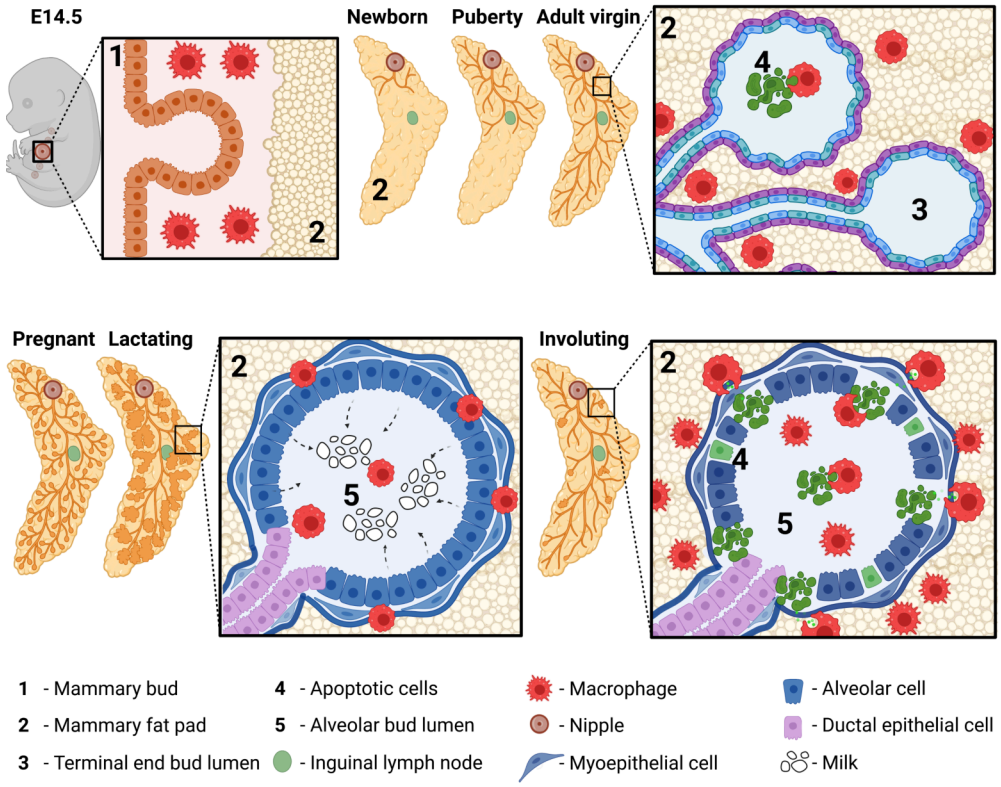

Figure 5 Mammary gland developmental stages and macrophage location. The mammary bud starts forming at E12.5 and macrophages were found to be close to the female mammary epithelium but not interacting with it. At birth, the mammary gland is only a rudimentary ductal structure, and the gland's growth stays quiescent until the onset of puberty. During pubertal mammary growth macrophages were described to be adjacent to the terminal end bud's head and also its neck, promoting the branching of the ducts. The mammary ductal network expands until it reaches the edge of the fat pad, with subsequent maturation of the gland. During pregnancy, new ductal branches grow and alveolar buds are formed. Before delivery, the alveoli mature and become capable of producing milk. During this stage macrophages are close to the basal cells, and also in the alveolar lumen, being able to leave the gland with the milk. After weaning, the alveoli regress and macrophages have been implicated in the process of clearing cell debris (Paine and Lewis 2017; Stewart et al. 2019). Created with BioRender.com

2.2.2 MG tissue resident macrophages

Several immune cells populate the MG stroma, such as mast cells, neutrophils, eosinophils and macrophages. Macrophages are involved in most of the MG's developmental stages, being crucial for the healthy growth of the gland (Reed and Schwertfeger 2010). Our study, published in 2019, revealed that macrophages are present in the MG already during embryonic development (see **Publication I**), opposing the notion that macrophages seed the MG stroma from 2 weeks after birth

onwards (Chua et al. 2010; Gouon-Evans, Rothenberg, and Pollard 2000). Later in 2019, another group using a Csf1r-EGFP mouse model studied the distribution of macrophages in mammary stroma throughout the different stages of development of this tissue (Stewart et al. 2019). Interestingly, macrophages were documented to seed the mammary mesenchyme as early as E14.5, and were found to be positioned adjacent to the embryonic mammary epithelium, although rarely interacting with female mammary epithelium. However, when the male ductal mammary bud starts to regress, macrophages were shown to invade the epithelium, possibly clearing the apoptotic cells. After birth, macrophages position adjacent to the terminal end of the bud's head and also its neck, between luminal and basal cells of the ductal epithelium. Additionally, macrophages were morphologically plastic during lactation, resembling alveolar basal cells adjacent to their location (Stewart et al. 2019). This data suggests a possible regulatory role for macrophages throughout all MG developmental stages, see **Figure 5**.

In fact, several studies have implicated macrophages as functional players in postnatal mammary morphogenesis: macrophages in the MG have a key role in the process of ductal elongation and branching (Gouon-Evans et al. 2000). Epithelial duct -associated macrophages regulate stem cell activity, constituting part of the mammary stem cell niche (Gyorki et al. 2009); during the involution phase M2-like macrophages are recruited by the involution microenvironment, suggesting a role in collagen clearance and organization (O'Brien et al. 2010). Macrophages are responsive to the estrous cycle, shifting their phenotype according to the dominant hormone (Hodson et al. 2013) and additionally, macrophages present a dual role in the epithelial cellular turnover. After ovulation they promote the growth the alveolar buds, and during anestrus they remodel mammary epithelium preparing for a new cycle (Chua et al. 2010). Epithelial duct -associated macrophages contribute to epithelial cell death and clearance, as well as to adipocyte repopulation, being crucial for tissue remodeling during the involution phase (Dawson et al. 2020; O'Brien et al. 2012).

A recent study elegantly described, by flow cytometry, several clusters of mammary macrophages defined by different cell markers: $CD11b^{+}F4/80^{+}$ (which increase and decrease in numbers according to the MG developmental stage), $F4/80^{+}CD206^{+}$, $F4/80^{+}CD11c^{+}$MHC II^{+} (defined as ductal macrophages, during lactation and involution comprise 20% of all leukocytes), and three other subpopulations of macrophages plus two different subsets of monocytes (Wilson et al. 2022).

Altogether, these data advocate for the existence of diverse subsets of mammary tissue resident macrophages either associated to the epithelium or to the stroma, having different functions in response to numerous stimuli. It is clear that a healthy development of the MG is a complex crosstalk between several cell types. Thus, a

small disturbance in these interactions might culminate in the onset of neoplasia. Macrophages play a key role in the regulation of MG morphogenesis, but the phenotype of the different subsets present in this tissue is not entirely established. Understanding the origin and function of MG macrophages is an important step towards a possible therapy for different mammary neoplasia.

2.2.2.1 Mammary macrophages as therapeutic targets

The MG is a very dynamic organ that is in constant development and remodeling, relying on several regulatory mechanisms, endocrine, paracrine and autocrine, to maintain a healthy function. These aspects are important for mammary physiology, but unfortunately make this organ vulnerable to the development of neoplasia. In fact, breast cancer has the highest incidence worldwide, and is the 6th leading cause of death by cancer (Sung et al. 2021). Several treatment approaches are being used, like hormonal blockage, surgery, radio and chemotherapy, however the mortality rate of breast cancer remains a concerning issue, demonstrating that new treatment strategies are required.

A recent study described murine MG tissue resident macrophages as a major source of tumor-associated-macrophages (TAMs) in a specific type of breast cancer (triple negative). In addition, the study suggested that targeting these resident macrophages can suppress early local recurrence after surgery and distant metastasis. Depleting the resident macrophages with clodronate blocked initial angiogenesis and disturbed the establishment of the tumor microenvironment (Hirano et al. 2023). Blocking TAMs recruitment has been an efficient way of studying macrophages functions during the settlement and development of breast cancer. For example, blocking the recruitment of CCR2^{+} monocytes to the mammary tumor using a CCL2-neutralizing antibody, leads to a reduction in macrophage infiltration, angiogenesis rate and formation of lung metastasis. However, unfortunately, if the antibody treatment was discontinued there was an exponential increase in metastasis (Bonapace et al. 2014), as the CCL2 concentrations after treatment were shown to surpass the ones measured before the treatment (Sandhu et al. 2013; Zhan et al. 2023). These data indicate that new immunotherapy targets need to be studied, and extensive phenotypic characterization of macrophages should be done, increasing the specificity of the treatment to avoid the risk of a negative rebound.

2.3 White Adipose Tissue (WAT)

Both invertebrates and vertebrates store energy in the form of fat. White adipose tissue (WAT) in mammals is not only a site for fat storage but also for the production of regulatory factors that affect the general function of the organism.

In Western society, food availability is constant, rendering the purpose of storing energy for times of scarcity obsolete. Obesity is defined as the abnormal accumulation of fat that associates with health risks like type 2 diabetes (T2D), cardiovascular disease and cancer. Notably, in the 1990's, obesity was already considered a pandemic by the World Health Organization (WHO) (Anon 1997), and unfortunately, the incidence of obesity is still rising. As the main phenotype of obesity is an expansion of white adipose tissue fat pads, studying this tissue development, growth, and function is important to understand the pathophysiology of obesity.

2.3.1 Morphology and location

The functional unit of WAT is the white adipocyte. This tissue also has a stromal vascular fraction (SVF) composed of pre-adipocytes, endothelial, neuronal cells, leukocytes, vascular progenitors, fibroblasts, and mesenchymal stem cells. The white adipocyte is a round cell containing a single lipid droplet that occupies around 90% of the cell, pushing the nucleus and the other cellular constituents close to the cellular membrane (Suzuki et al. 2011). Adipocytes are very plastic cells, their diameter can range from 20µm (new adipocyte) to 120µm (obese adipocyte) in mice (Li and Spalding 2022).

White adipose tissue is the most abundant type of fat in the body, and it is a multi-depot organ. The main depots existing in humans are: subcutaneous, visceral, and bone marrow fat (Zwick et al. 2018). The visceral adipose tissue has a strong association with the development of metabolic diseases. The visceral fat depot is composed of omental, mesenteric, retroperitoneal, perigonadal, and pericardial fat pads (Rosen and Spiegelman 2014). While mouse fat depots do not exhibit a perfect correlation with human depots, they do share several noteworthy similarities with human WAT. Despite the existence of a comparable fat depot in humans, murine gonadal fat pads are frequently used in research due to their larger size and easy accessibility (Chusyd et al. 2016). Additionally, adipose tissue fat pads have great gender variability in location, adipose depot mass, and functionality. These differences can be associated with the effects of sex hormones (Fuente-Martín et al. 2013). Obesity research primarily uses male mice due to their susceptibility to obesogenic diets, while female mice are often considered resistant (Huang et al. 2020; Maric et al. 2022).

2.3.2 Function and development

WAT is a site for energy storage in the form of fat, and may function as a protective cushion for internal organs. Moreover, WAT regulates whole body energy

homeostasis by storing and releasing lipids in accordance with the organism needs. The lipids are stored in the form of triglycerides inside the adipocytes (Smorlesi et al. 2012). Importantly, WAT is considered the largest endocrine organ. By secreting numerous factors such as hormones, lipids, and cytokines (adipokines), WAT controls the appetite, lipid metabolism, glucose homeostasis, insulin sensitivity, and many other functions (Choe et al. 2016).

WAT secretes a wide range of factors, such as leptin controlling the satiety axis, and adiponectin that is negatively correlated to insulin resistance (Nguyen 2020; Obradovic et al. 2021; Torres, Vargas-Castillo, and Tovar 2016; Trayhurn and Beattie 2001; Vázquez-Vela, Torres, and Tovar 2008). Additionally, WAT produces different cytokines that enable the polarization of different subsets of macrophages, regulating the physiological and pathophysiological development of the tissue (Pan et al. 2023).

2.3.2.1 Prenatal development

WAT fat pads are forming around birth in mice and around 14 weeks of gestation in humans. Fat deposition follows the growth of the body throughout the whole lifespan (Wayne et al. 2020). The developmental origin of white adipocytes is still under study, as it is a complex process involving different pathways. Adipocytes generally emerge from mesenchymal stem cell precursors, from intermediate and lateral plate mesoderm (Berry et al. 2013). When exposed to retinoic acid, embryonic stem cells from the neural crest can also commit to adipogenic lineage (Yao, Dani, and Dani 2022). *Wt1* (Wilm's tumor gene) is expressed in visceral but not in subcutaneous white pre-adipocytes, suggesting a distinct ontogeny between fat depots. However, *Wt1* expressing precursors only contribute partially for the visceral white adipocyte pool, indicating the presence of heterogeneous adipocyte lineages (Chau et al. 2014). $Myf5^+$ (myogenic factor 5) and $Pax3^+$ (paired box 3) precursors are known to follow the myogenic and brown adipocyte lineage. Still, white adipocytes from subcutaneous and retroperitoneal WAT depots may differentiate from $Myf5^+$ and $Pax3^+$ mesenchymal precursor cells (Sanchez-Gurmaches, Hung, and Guertin 2016). Thus, white adipocytes can have multiple origins, and a specific lineage marker has yet to be identified.

Mesenchymal stem cells differentiate into adipoblasts, a common early precursor committed to the adipocyte lineage. Adipoblasts develop into pre-adipocytes that populate a specific perivascular niche within the fat depot (Gesta, Tseng, and Kahn 2007). Adipogenesis is the process of differentiation from pre-adipocytes to adipocytes. PPARγ (peroxisome proliferator-activated receptor gamma) is the master regulator of adipogenesis, functioning in combination with members of the C/EBPs (CCAAT-enhancer-binding proteins) family (Farmer 2006). PPARγ also

has a critical role in pre-adipocyte self-maintenance (Berry et al. 2013). Pre-adipocytes can be identified around E16.5 by their PPARγ, perilipin, and adiponectin expression and highly active proliferation rates (Hong et al. 2015).

2.3.2.2 Postnatal development

Postnatal growth of the fat depots occurs not only by differentiation of pre-adipocytes into new adipocytes (hyperplasia) but also by increasing the size of the adipocytes (hypertrophy). Interestingly, several studies have described adipocyte hypertrophy as being usually associated with several risk factors for the development of metabolic and cardiovascular disorders (Honecker et al. 2022; Laforest et al. 2015; Skurk et al. 2007; Suárez-Cuenca et al. 2021; Weyer et al. 2000). Adipocyte expansion by hyperplasia starts already before birth, and is essential to the initial development of the depots, but stabilizes early in life (Li and Spalding 2022). Hypertrophic growth is regulated by the balance of two physiologic processes, lipogenesis, and lipolysis. Lipogenesis is the triglyceride synthesis from fatty acids and glucose. Lipolysis is the triglyceride break down into glycerol and three molecules of fatty acids. Both processes are controlled through different pathways, such as the insulin pathway that promotes lipogenesis while inhibiting lipolysis (Li and Spalding 2022). Adipocyte growth is limited. When the cell reaches its maximum volume, the lipids are stored in ectopic locations, such as the liver (in hepatocytes) and muscle (as intramyocellular lipids), or even phagocytosed by macrophages (foamy cells). This phenomenon of "lipotoxicity" significantly affects metabolic homeostasis, particularly glucose metabolism, and is widely considered a key contributor to the development of metabolic disorders (Arner and Rydén 2022).

WAT is highly plastic, requiring high vascularization for its growth. Hence, the WAT expansion is angiogenesis-dependent. The capillaries that supply adipose tissue play a crucial role not only in delivering nutrients, oxygen, cytokines, growth factors, or hormones but also transporting the substances produced by adipocytes. Importantly, angiogenesis regulates the microenvironment of WAT, affecting adipocyte function, pre-adipocyte differentiation, and overall adipose tissue mass. In an obese state, angiogenesis is impaired, leading to tissue hypoxia and subsequent recruitment of macrophages (Herold and Kalucka 2021). Angiogenesis in the WAT is regulated by angiogenic adipokines together with vascular endothelium factors such as VEGFA (vascular endothelial growth factor A). Leptin and adiponectin interfere by upregulating or inhibiting angiogenesis, respectively (Cao 2010).

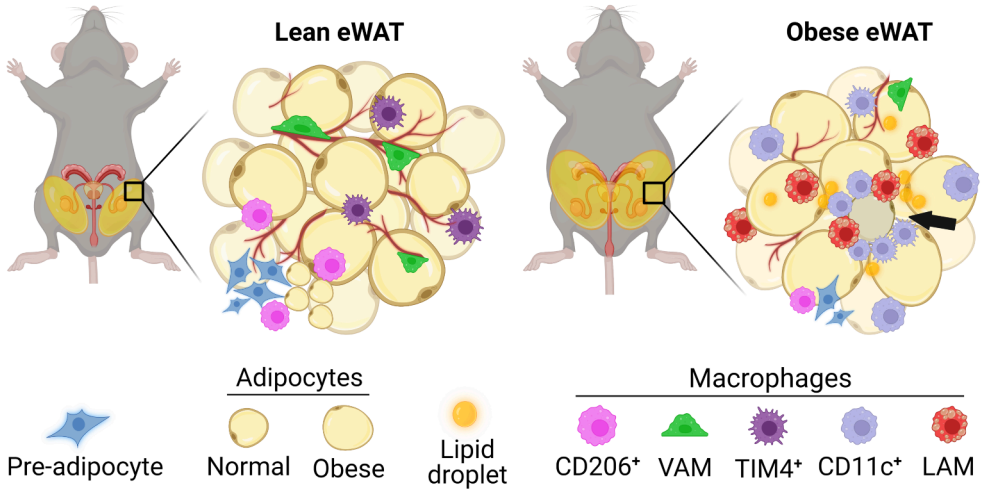

Figure 6 Representative figure of white adipose tissue (WAT) macrophages in lean and obese states. Lean epididymal (e)WAT is populated by different macrophage populations. $CD206^+$ macrophages interact with the pre-adipocytes and regulate adipogenesis. Vasculature associated macrophages (VAMs) co-localize with vessels and readily uptake particles from circulation. $TIM4^+$ macrophages are embryonic-derived, and regulate lipid storage in adipocytes. In obese eWAT, adipocytes grow in size and become dysfunctional not being able to uptake more lipids. Lipid associated macrophages (LAMs) are recruited to prevent hypertrophic growth of adipocytes, in early-stage obesity. Concomitantly, there is an influx of $CD11c^+$ inflammatory macrophages that populate regions of apoptosis forming crown-like structures (black arrow), together with LAMs. Created with BioRender.com

2.3.3 White adipose tissue resident macrophages

Macrophages associated with the WAT have been extensively studied in the context of obesity. The obese state in WAT is characterized by an infiltration of macrophages (Daemen and Schilling 2020; Korf et al. 2019; Weisberg et al. 2003; Zamarron et al. 2017). These macrophages are the main players in the progression of metabolic syndrome and the chronic low-grade inflammation typical in obese individuals (Cai et al. 2022; Ni et al. 2020). However, not all macrophages involved in the pathophysiology of obesity are recruited from bone marrow. Adipose tissue resident macrophages, ATMs henceforth, have a pivotal role in regulating WAT function and responding readily to local stimuli, being the first to proliferate in an obese context (Amano et al. 2014; Zheng et al. 2016).

In a steady state, macrophages are already the predominant immune cell within WAT. During the progression of obesity, 50% of the total cells of the tissue can be composed of macrophages (Ferrante 2013). ATM origin has been under study, and it has been verified that some of the populations existent in a steady state originate from the embryonic period (Waqas et al. 2017). Macrophages in murine WAT are usually identified as $CD11b^+$ $F4/80^+$ $CD64^+$. Most ATMs in a steady state have an

anti-inflammatory profile, and express a $CD206^+$ membrane marker. However, macrophages infiltrating the tissue during the onset of obesity usually express a CD11c membrane marker, due to their pro-inflammatory profile, see **Figure 6** (Caslin et al. 2020; Ferrante 2013; Korf et al. 2019; Thomas and Apovian 2017).

Embryonic-derived ATMs have been described as the major contributors to the macrophage pool of lean WAT (Waqas et al. 2017). Interestingly, these long lived and self-maintaining macrophages are defined by TIM4 (T Cell Immunoglobulin And Mucin Domain Containing 4) cell membrane marker expression (Chen and Ruedl 2020; Cox et al. 2021). Cox et al. 2021 demonstrated that embryonic-derived $TIM4^+$ ATMs produce PDGFcc (platelet-derived growth factor cc), which regulates lipid storage in adipocytes. By increasing PDGFcc secretion, ATMs improve the lipid storage capacity of adipocytes, decreasing ectopic fat deposition.

An ATM population defined by its CD206 expression, is crucial for the maintenance of the pre-adipocyte niche, by inhibiting the adipogenesis rate in a TGFβ-dependent manner. Additionally, the depletion of $CD206^+$ macrophages had a browning (white to brown-like adipose tissue) effect in the adipose tissue, attributed to a lack of inhibition in the differentiation of brown pre-adipocytes (Nawaz et al. 2017; Nawaz and Tobe 2019).

Vascular associated macrophages (VAMs) also express CD206 and have been described in both lean and obese adipose tissue. VAMs are in close contact with the vessels, having a high endocytic capacity, and keeping adipose tissue clear of catabolites from lipogenesis/lipolysis. VAMs are suggested to be self-maintaining and not originating from bone marrow progenitors. Furthermore, VAMs' phagocytic capacity is suspended in response to nutritional (obesity) or infection (LPS) stress (Silva et al. 2019).

In an obese state, macrophages are found in crown-like structures, that surround dying/defective adipocytes, promote apoptosis and resorb lipids (Murano et al. 2008). TREM2 (triggering receptor expressed on myeloid cells 2) signal leads to the recruitment of lipid-associated macrophages (LAMs) in crown like structures in early state obesity (Stansbury et al. 2023). LAMs prevent the hypertrophic growth of adipocytes, having a gene expression signature associated with phagocytosis and lipid catabolism. Additionally, LAMs are thought to regulate the immune response due to the expression of immunosuppressor genes (Jaitin et al. 2019). The TREM2 pathway has also been associated with insulin sensitivity (Tanaka et al. 2018), adipogenesis, and weight gain (Park et al. 2015).

Adipocyte hypertrophy leads to the secretion, by adipocytes, of IL-6, IL-8, monocyte chemoattractant protein (MCP1), and leptin. Obese adipocytes have an inflammatory gene signature, and also have defects in glucose transporter type 4 (GLUT4), leading to insulin resistance (Li and Spalding 2022). Macrophages chemoattracted to adipose tissue during the onset of obesity are known to be

dependent on the CCR2/CCL2 axis (Weisberg et al. 2006). Blocking the CCR2/CCL2 axis leads to a significant resistance to weight gain, but does not completely revert it. Nonetheless, this therapy reduced the inflammatory state associated with the obese state, and consequently ameliorated the symptoms of insulin resistance (Kim et al. 2016).

ATMs are crucial for the homeostasis of WAT, as they are involved in many regulatory mechanisms. However, due to different research approaches a consensus view has not been reached regarding macrophage subtyping. Thus, a unifying study of the different macrophage subsets in WAT might trigger the discovery of new anti-obesity targets.

2.4 Brown Adipose Tissue

As obesity becomes increasingly prominent in the Western world, several different approaches to treat and prevent it are being studied. Brown adipose tissue (BAT) has become a significant research interest in fighting obesity, after being described as an active metabolic organ in adult humans in 2009 (Cypess et al. 2009; van Marken Lichtenbelt et al. 2009; Virtanen et al. 2009). Historically, BAT was believed to remain solely active in hibernating animals and human infants, diminishing in size and metabolic capacity as postnatal life progressed. It is essential to notice the existence of brite/beige adipose tissue, which also has thermogenic activity, being an intermediate tissue between white and brown adipose tissues (Cheng et al. 2021). However, its functionality and development are not within the scope of this doctoral dissertation.

BAT brings important implications for the development of new treatments for obesity and related metabolic disorders, as well as for understanding the complex mechanisms involved in energy homeostasis in the body.

2.4.1 Morphology and location

The functional unit of BAT is the brown adipocyte. Its coloration is associated with the abundance of mitochondria in their cytoplasm. Brown adipocytes differ from white adipocytes in mitochondria density and in the lipid droplets existing within them. Brown adipocytes have small multilocular lipid droplets (Cinti 2005; Richard and Picard 2011), contrasting with the single lipid droplet characteristic of white adipocytes described previously. In newborn humans, BAT is found in several locations and can be divided into visceral BAT and subcutaneous BAT. However, with aging, this tissue disappears, and in adults, metabolic active BAT fat pad is highly variable and is mainly found in the supraclavicular fossa and cervical region (Cypess et al. 2009; Frontini and Cinti 2010; Sacks and Symonds 2013; Zhang et al.

2018). BAT is studied in mice, taking advantage of murine BAT's high metabolic activity and well-defined fat pads. The major BAT fat pads in mice are found in the dorsal region, mainly in between the shoulder blades (interscapular BAT – iBAT), and other pads distributed in the supraclavicular, anterior cervical, axillary, supraspinal, ventral spinal, and perirenal regions (Cai and He 2023; Cinti 2005; Frontini and Cinti 2010; de Jong et al. 2015; Waldén et al. 2012; Zhang et al. 2018), see **Figure 7**.

BAT is a site for non-shivering thermogenesis, which means that instead of just storing lipids, like WAT, this tissue has the capacity to use the lipids to produce heat; this is crucial in the process of thermoregulation during the perinatal phase. Its fat-burning ability is not the only capability of interests to investigators in the field of obesity research. BAT is also an active metabolic organ, being responsive to insulin and other metabolic cues, and secreting brown adipokines (batokines) with endo-, para- and autocrine roles (Villarroya et al. 2016).

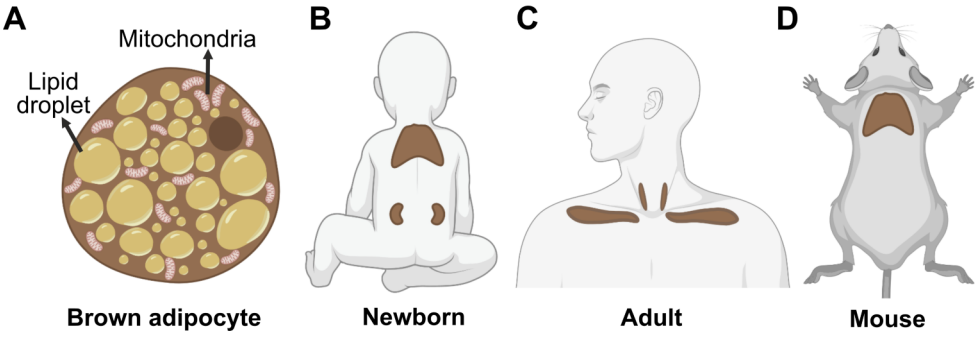

Figure 7 Brown adipocyte structure and major BAT depots. **A**. Note the multilocular lipid droplets and the abundancy of mitochondria within a brown adipocyte. **B**. Human infants have large interscapular and perirenal BAT depots. **C**. BAT regresses with aging, and in adults the most common brown fat pads found are in the clavicular and cervical region. **D**. In mice, the major BAT depot is located in the interscapular region. Created with BioRender.com

2.4.2 Function

BAT regulates systemic energy homeostasis through non-shivering thermogenesis, enabling the adjustment to colder environments by increasing BAT's metabolic activity rate (Ikeda and Yamada 2020; Richard and Picard 2011). BAT thermogenesis is mainly measured by quantifying the presence of uncoupling protein 1 (UCP1), found in the inner membrane of mitochondria in brown adipocytes. During oxidative phosphorylation or mitochondrial respiration, the proton gradient created in the mitochondria's inner membrane is used for the generation of heat instead of ATP, using triglycerides as fuel. Interestingly, for a long time, it was

believed that BAT thermogenesis was only UCP1-dependent (Ricquier 2011). However, different compensatory metabolic pathways independent of UCP1 exist, like creatine-substrate cycling or Ca^{2+} cycling (Ikeda et al. 2022; Ikeda and Yamada 2020; Rahbani et al. 2022). Generally, the thermogenic potential of BAT intensifies not only with the increase in the quantity of UCP1, by the production of more protein, or by mitochondrial proliferation but also with the generation of new brown adipocytes. The loss of UCP1 significantly diminishes BAT thermogenic activity and is associated with increased body mass, proving its importance to the functionality of the tissue and BAT's crucial role in obesity (Feldmann et al. 2009; Lowell et al. 1993).

The sympathetic nervous system (SNS) has a critical function in regulating BAT activity, activated by cold exposure, exercise, food consumption, and several hormones and cytokines (Coolbaugh et al. 2019; Huo et al. 2022; Nedergaard and Cannon 2022; Peres Valgas da Silva et al. 2019; Quarta et al. 2012; Richard and Picard 2011; Saito et al. 2020; Zekri et al. 2022). Adaptive thermogenesis is a mechanism that is regulated in several brain regions belonging to the autonomic brain system (hypothalamic and brain stem circuits) (Morrison and Madden 2014; Muzik and Diwadkar 2017; Richard and Picard 2011). In cold environments, lower temperatures are detected in the skin receptors. This information is directed to the hypothalamus (regulator center for the body temperature), which forwards the signal to the peripheral sympathetic nervous fibers deeply rooted within BAT. Noradrenaline is released by the fibers, stimulating the β3-adrenergic receptors in brown adipocytes to initiate adaptive thermogenesis to produce heat. Thus, BAT adapts to the environmental temperature, responding rapidly when the animal is subjected to a cold or warm period. The thermoneutral temperature for mice is around 30°C (Gordon 2012, 2017; Trayhurn and James 1978; Vialard and Olivier 2020), which is usually not maintained in a research environment, as mice are typically housed at 21°C. When housed at 30°C, the murine brown adipocytes lose their mitochondria and multilocular lipid droplet phenotype, and start to resemble white adipocytes, even at the gene expression level. However, if these animals are challenged with cold, the phenotype is rescued (Davies et al. 2023), indicating that BAT is a highly plastic tissue, able to adapt quickly to external cues.

2.4.2.1 BAT as a metabolic organ

BAT is considered a metabolic organ, as it responds to insulin, and it is a known glucose uptake site, as well as a site for triglyceride clearance. Moreover, brown adipocytes secrete hormones and batokines that regulate metabolism, including adiponectin and leptin, fibroblast growth factor 21 (FGF21), IL-6, and CXCL14 (C-X-C motif chemokine ligand-14) (Shinde, Song, and Wang 2021).

Functional BAT is associated with less complications of a positive metabolic imbalance (excess energy intake), both directly by improving metabolic rate and burning the excess of lipids and indirectly by being sensitive to insulin and a glucose-clearing site. Active BAT can contribute to 2-5% of the basal metabolic rate in humans (van Marken Lichtenbelt and Schrauwen 2011; Porter, Chondronikola, and Sidossis 2015), and it has been associated with a lower body mass index, alleviating insulin resistance and keeping the glucose homeostasis. BAT fully oxidizes glucose, not in the process of thermogenesis *per se*, but to replenish the triglycerides storage within the adipocytes, and this mechanism is believed to be insulin-stimulated (Orava et al. 2011). Several studies have proven that BAT plays a crucial role in the amelioration of obesity symptoms and in whole-body energy metabolism. BAT's de-innervation or ablation increases the triglyceride concentration in blood and significantly increases body fat (Dulloo and Miller 2011; Lowell et al. 1993). Contrastingly, BAT transplantation has been proved to reverse weight gain, reduce obesity-associated adipose tissue inflammation, and improved insulin sensitivity (Liu et al. 2015, 2023; Zhu et al. 2014).

Several batokines have been described to intervene in whole body metabolism and general BAT function (Ahmad et al. 2021; Villarroya et al. 2016; Yang and Stanford 2022). For example, BAT requires increased thermogenesis in a cold environment, so as to stimulate tissue growth by hyperplasia and/or hypertrophy. As the tissue grows and remains highly active, there is the need for angiogenesis, guaranteeing sufficient blood supply. BAT secretes VEGFA, promoting angiogenesis and upregulating UCP1. Moreover, chemerin production is upregulated in obesity, increasing lipid accumulation and serving as a chemoattractant for immune cells, particularly for macrophages. Both VEGFA and chemerin are autocrine and paracrine factors. Additionally, BAT can produce several endocrine factors, such as FGF21, which is expressed in activated brown adipocytes and improves hyperglycemia and hyperlipidemia and, IL-6, which promotes insulin sensitivity and induces the browning of WAT; IL-6 is also produced upon β-adrenergic stimulation of brown adipocytes (Villarroya et al. 2016). Another endocrine batokine is CXCL14, which is produced in response to cold-stimulation of brown adipocytes, and has beneficial effects on insulin sensitivity. Moreover, it facilitates the polarization of anti-inflammatory macrophages to WAT fat pads, promoting the browning of white adipocytes (Cereijo et al. 2018). Cross talk between brown adipocytes and macrophages has been widely proven. GDF15 (growth and differentiation factor 15) secreted by brown adipocytes in the context of thermogenic activation, represses the pro-inflammatory expressed genes in macrophages (Campderrós et al. 2019).

2.4.3 BAT development

Interestingly, brown adipocytes were initially thought to originate from the same multipotent mesenchymal stem cells as white adipocytes, having PPARγ as a key player in the differentiation of both lineages (Rosen et al. 1999; Symonds 2013). However, BAT differs from WAT, as it originates from precursors cells that can also differentiate into skeletal muscle and dermis. BAT precursor cells can express Myf5 and/or Pax7, indicating that brown adipocytes might have multiple origins (Timmons et al. 2007). Nevertheless, brown pre-adipocytes maintain a myogenic gene expression signature which is lacking in white pre-adipocytes, suggesting a shared origin with muscle progenitor cells (Timmons et al. 2007). Furthermore, PRDM16 (PR domain zinc-finger protein 16) was recognized as a critical regulator for the switch to brown adipocytes instead of skeletal muscle (Seale et al. 2008; Shinde et al. 2021).

Differentiation of brown adipocytes occurs in the murine embryo around day E10-12.5, but only on E15.5, the depot is clearly located in the interscapular region (Mayeuf-Louchart et al. 2019; Shinde et al. 2021). UCP1 production is barely identifiable during these stages, peaking right before birth (Houštěk et al. 1988; Liu et al. 2020; Shinde et al. 2021). At E17.5, brown adipocytes are functionally differentiated (Mayeuf-Louchart et al. 2019). The heterogeneity of brown adipocytes in iBAT reaches its peak around two weeks after birth, with a lower and higher thermogenic capacity of the brown adipocytes (Shinde et al. 2021; Song et al. 2020).

Before birth, the uterine environment offers the embryos a consistent, warm environment. At this point, BAT mainly develops through hyperplasia and hypertrophy, without upregulating thermogenic associated genes (Kumagai, Saito, and Kida 2023; Liu et al. 2020), like UCP1, DIO2 (Iodothyronine Deiodinase 2) (Jesus et al. 2001) and PGC1α (regulates mitochondria biogenesis, adaptive thermogenesis, and oxidative metabolism - peroxisome proliferator-activated receptor-gamma coactivator alpha) (Liang and Ward 2006). Interestingly, in altricial species (born immature), such as mice, the hypothalamic-pituitary-adrenal axis only fully matures postnatally, which might delay a full response from non-shivering thermogenesis in BAT, leading to the "nesting close together to keep warm" behavior in mice pups (Cannon et al. 1988; Symonds, Pope, and Budge 2015). At birth and during the first weeks of development, BAT's contribution to the energy balance is crucial in most species. With the ingestion of maternal milk, there is access to lipids used as energy for thermogenesis via the PPARα pathway, which promotes fatty acid catabolism and is already activated a few days before birth. Additionally, PPARα also stimulates the production of FGF21 in the liver, which increases the expression of thermogenic genes in BAT (Hondares et al. 2010; Rando et al. 2016). During postnatal life, the brown adipocytes grow in size as the triglyceride stocks increase, expanding the lipid droplet volume; mitochondria volume also increases,

although their numbers drop. UCP1 and PPARα expression decreases throughout aging, but there is an upregulation peak at 2 and 4 weeks for other thermogenic genes, like PGC1α (Liu et al. 2020). A recent study accounted for two major shifts in BAT metabolome in a steady state from late embryonic development to adult life in mice. The first shift is set at birth when BAT starts using lipids as fuel instead of stored energy in ATP or GTP form. The second shift is seen around four weeks, and is associated with the weaning age, and with the high stimulation of neurons related to feeding and energy expenditure control (Kumagai et al. 2023). These data show that from embryonic development until weaning age, ~4 weeks old, murine BAT goes through many morphological, transcriptomic and metabolomics changes essential for tissue function in a mature state.

2.4.4 BAT resident macrophages

BAT comprises other cell types apart from brown adipocytes; immune cells are one of them. Several studies have focused on the role of tissue-resident macrophages in BAT in the context of induced obesity, guided by the established correlation between macrophages in white adipose tissue and the nefarious effects of inflammation in an obese-state. In adult BAT, in a steady state, macrophages have been shown to constitute only a small portion of the whole tissue and around 30% of all immune cells (Peterson, Flaherty, and Hasty 2017). *In vitro*, brown adipocytes co-cultured with macrophages induced downregulation of the inflammatory profile (Dowal et al. 2017). When challenging lean and obese animals with cold, BAT adaptive thermogenesis is not impaired in either of the groups. However, only lean individuals show an increase in anti-inflammatory macrophage numbers, suggesting that macrophages do not interfere in the process of UCP1 upregulation (Boulet et al. 2021). In contrast, at thermoneutrality (30ºC), there is a clear infiltration of macrophages into BAT, which mainly localize in crown-like structures, similar to those described in obese WAT. Nevertheless, this macrophage infiltration occurs regardless of lean or obese states. Interestingly, this phenotype was rescued when the mice were re-acclimated to cold, and no BAT functional impairment was detected (Fischer et al. 2020). BAT macrophages were described as catecholamines producing cells, having a direct influence on thermogenesis (Nguyen et al. 2011). However, this role was contested later by a paper where six different laboratories showed that macrophages are not able to express tyrosine hydroxylase (TH) and could not produce the relevant amounts of catecholamines to induce thermogenesis (Fischer et al. 2017).

To date, flow cytometry studies have used the F4/80 and CD64 expression to identify BAT macrophages, but, as discussed earlier, tissue resident macrophages are usually a heterogenic population composed of different subsets of macrophages

that differ in function, location, and origin. Not much is known about the different subsets of brown adipose tissue resident macrophages. In 2017, *Wolf et al.* described BAT as having several subsets of macrophages defined by CX3CR1 expression. Much like microglia, $CX3CR1^{+}$ macrophages were associated with SNS fibers existing in BAT, which play an essential role in the maintenance of the sympathetic innervation of brown adipocytes and, consequently, in thermogenesis (Wolf et al. 2017). In 2021 *Gallerand et al.* performed scRNA sequencing of steady state and genetically-induced obese murine BAT leukocytes. They revealed the existence of several macrophage and monocyte subsets in both healthy and obese BAT, with a substantial increase of bone marrow-derived monocyte/macrophage CCR2-dependent recruitment in the latter. Interestingly, the influx of these cells did not lead to the upregulation of inflammatory markers but was hinted to have an important role in tissue homeostasis supporting tissue remodeling (Gallerand et al. 2021). Another subset of macrophages associated with sympathetic neuronal fibers was described in subcutaneous adipose tissue and BAT. These macrophages express solute carrier family 6 member 2 (Slc6a2) and are able to dispose of catecholamines existing in the microenvironment. This subset was increased in the obese state, indicating a possible pro-inflammatory action (Pirzgalska et al. 2017).

BAT macrophages seem to play an essential role in tissue homeostasis and in assisting thermogenesis, at least indirectly, by regulating SNS stimulation. Accordingly, further understanding of the different macrophage subsets existing in functional BAT might present a target to potentiate the beneficial features of the BAT function to the metabolism of the whole body, thus preventing obesity.

3 Aims

Every tissue has its unique tissue resident macrophages. These macrophages are involved in the regulation of morphogenesis, tissue homeostasis and remodeling, metabolism, healing reactions, and even cancer progression. Whether the developmental origin of tissue resident macrophages has a significant effect on the functionality of these cells is still unsolved. This thesis hypothesizes that embryonic-derived tissue resident macrophages have a crucial role in the formation, development and function of the MG, WAT and BAT. Thus, the aims of this study are:

1. To unravel the landscape and origins of macrophage subsets in steady state MG, WAT and BAT.
2. To understand the role of embryonic macrophages and their contribution to the development and function of MG, WAT and BAT.
3. To study how obesity and weight loss affect the resident macrophage subpopulations in WAT.

4 Materials and Methods

This dissertation is based on two original publications and a manuscript, which are referred to in the text by the Roman numerals I, II, and II, respectively, see **List of Original Publications**. All reagents used are listed in **Table 8** at the end of the Material and Methods section.

4.1 Animals (I-III)

All the murine (*Mus musculus*) strains used during the course of the studies were housed in the animal facilities of the University of Turku (Turku, Finland) under controlled specific pathogen free conditions and a 12h light/dark cycle at a room temperature (RT) of 22ºC± 1ºC, unless stated otherwise. The animals were fed with standard pellet chow and reverse osmosis water *ad libitum*, except otherwise specified. Embryonic development was estimated by considering the day of a vaginal plug as embryonic day 0.5 (E0.5). Unless stated otherwise, the tissues collected were: the 4th MG from virgin females (I), gonadal white adipose tissue from males (II – epididymal WAT) and interscapular brown adipose tissue from males or females (III). The animals were used at the indicated age and sex. When genetically modified mouse models were studied, age and sex matched WT or littermates were used as controls.

These studies were performed in agreement with the regulations of the Regional Animal Experiment Board in Finland, following the 3R principle and according to the Finnish Act on Animal Experimentation 541 (497/2013). Additionally, the experiments were conducted under animal license numbers 5587/04.10.07/2014, 6211/04.10.07/2017 and 540 14685/2020.

4.1.1 Tamoxifen inducible models

Several fate mapping studies were performed throughout the dissertation work. Generally, in these studies we fluorescently labeled a gene of interest by inducing reporter recombination in the offspring at specific time points, usually embryonic, which allowed the study of the fate of the cells that expressed that gene at the time of injection.

To label yolk sac-derived macrophages, two tamoxifen-inducible models were used:

1. *Csf1r*^{CreEsr1} males were crossed with *R26R-EYFP* females, and at E8.5 females were injected i.p. with tamoxifen (1.5 mg) and progesterone (0.75 mg). Tissue collection at newborn (I: Sup. Fig.2g) and 5 weeks (I: Sup. Fig.2g; II: Fig.3a).
2. *Cx3cr1*^{CreERT2} males were crossed with *R26R-EYFP* females, tamoxifen and progesterone injections were done as above in 1 at E9.5, with tissue collection at E17.5 (III: Sup. Fig. 2d) and 5 weeks (II: Fig.3c). Or E13.5 pulse with tissue collection at newborn, 2 and 5 weeks (I: Fig1b) or 5 and 10 weeks (II: Fig.3e).

To label fetal liver-derived macrophages, one tamoxifen-inducible model was used:

1. *Ccr2*^{CreERT2} mice were crossed with TdTomato^{lox} mice, and at E14.5 and E16.5, females were injected i.p. with tamoxifen (1.5 mg). Tissue collection at E17.5, 2, and 5 weeks (III: Fig. 2a).

To label bone marrow-derived macrophages, one tamoxifen-inducible model was used:

1. Ms4a3^{CreERT2} mice were crossed with TdTomato^{lox} mice, and at postnatal day 5, the progeny was injected i.p. with tamoxifen (0.56 mg). Tissue collection at E17.5, 2, and 5 weeks (III: Fig. 2c).

Table 1. Mouse strains used in the dissertation work.

STRAIN NOMENCLATURE		SOURCE	CAT. #	PUB.
Wild type				
C57BL/6JRj (Wild type)*	WT 6J	Janvier Labs		I-II
C57BL/6NRj (Wild type)	WT	Janvier Labs		I-III
Knock out				
B6.129S4-*Ccr2*^{tm1Ifc}/J	*Ccr2*$^{-/-}$	The Jackson Laboratory	004999	I-II
B6;129S2-*Nr4a1*^{tm1Jmi}/J	*Nur77*$^{-/-}$	The Jackson Laboratory	006187	I-II
B6;*C3Fe a/a-Csf1*^{op}*/J*	*Csf1*^{op}	The Jackson Laboratory	000231	I-II
Plvap^{tm1Salm}	*Plvap*$^{-/-}$	Rantakari *et al*., 2015		I-III

STRAIN NOMENCLATURE		SOURCE	CAT. #	PUB.
Tamoxifen inducible				
B6.129P2(C)-*Cx3cr1*$^{tm2.1(cre/ERT2)Jung}$/J	*Cx3cr1*^{CreERT2}	The Jackson Laboratory	020940	I-III
C57BL/6NTac-*Ccr2tm2982(T2A-Cre7ESR1-T2A-mKate2)BB*	*Ccr2*^{CreERT2}	Croxford, *et al.*, 2015		III
Ms4a3$^{tm1(cre/ERT2)Smoc}$	*Ms4a3*^{CreERT2}	Liu, *et al.*, 2019		III
Tg(Csf1r-mer-iCre-Mer)1jwp	*Csf1r*^{CreEsr1}	The Jackson Laboratory	019098	I-II
Fluorescent tracer (to cross with *cre*-expressing models)				
B6.129X1-*Gt(ROSA)26Sor*$^{tm1(EYFP)Cos}$	*R26R-EYFP*	The Jackson Laboratory	006148	I-II
B6.CG-*GT(ROSA)26SOR*$^{TM14(CAG-TDTOMATO)HZE}$/*J*	*TdTomato*^{lox}	The Jackson Laboratory	007914	III

* Wild type strain used as control for *Nur77*$^{-/-}$, because of its matching background. All other experiments were conducted with WT (C57BL/6NRJ), or control littermates.

4.2 Macrophage depletion

4.2.1 Depletion of yolk sac-derived macrophages during embryonic development (I)

Pregnant E6.5 WT mice were injected i.p. with 3mg of CSF1R blocking antibody to deplete yolk sac-derived macrophages of the offspring, or rat anti-IgG2 for control (Hoeffel et al. 2015; Squarzoni et al. 2014). Tissues were collected at E17.5, 2 or 5-week-old for flow cytometry.

4.2.2 Postnatal depletion of tissue resident macrophages (I)

2-week-old WT mice were sequentially injected i.p. with CSF1 antibody (0,5mg) followed by the next day 50µL i.v. clodronate administration, IgG and liposomes were administered as control, respectively. After 48h recovery the consecutive treatments were repeated cyclically 2 more times, having lowered the dosage of anti-CSF1 to half. Tissues were collected 24h or 12 days after the last clodronate injection.

Table 2. Reagents used for macrophage depletion.

	SOURCE	CAT. #	RRID	PUB.
Antibodies				
CSF1	Bio X Cell	BE0204	AB_10950309	I
CSF1R	Bio X Cell	BE0213	AB_2687699	I
IgG1	Bio X Cell	BE0088	AB_1107775	I
IgG2a	Bio X Cell	BE0089	AB_1107769	I
Liposomes				
Clodronate Liposomes	Liposoma research liposomes		clodronateliposomes.com	I
Control Liposomes	Liposoma research liposomes		clodronateliposomes.com	I

4.3 Cytometry

MG (without the inguinal lymph node), eWAT, and iBAT were collected in Hanks' Buffered Saline solution and processed by mechanical means (minced with scissors) and enzymatic dissociation (1 mg/ml collagenase D and 50 µg/ml DNase 1 – 1h at 37ºC) into a single cell suspension. After digestion, ≥5 week-old eWAT and iBAT required gravitational separation of SVF. All three tissues underwent sequential filtering through silk (77µm) washing and suspension in PBS before staining with fluorescent – flow cytometry and cell sorting – or metal tagged antibodies – mass cytometry. The list of antibodies and reagents used in the three publications are listed in **Table 3** and **Table 8**, respectively.

Control tissues were collected and used as a single cell suspension with different procedures, briefly: blood was collected through a cardiac puncture with heparin coated syringe (≥2-week-old mice). Erythrocytes were lysed by hypotonic lysis (I & III); Brains were mechanically and enzymatically (30' at 37ºC) dissociated, and microglia were isolated followed by a Percoll gradient (I-III) (Lee and Tansey 2013); Lung was mechanically dissociated with scissors followed by enzymatic digestion for 1h at 37ºC, filtered through a 70µm cell strainer, and through silk (III).

4.3.1 Flow cytometry (I-III) and cell sorting (III)

The single cell suspensions were incubated with eFluor780 viability dye for 30 minutes. Consequently, Fc-receptors were blocked with anti-CD16/32 for 10 minutes. A fluorochrome-conjugated antibody mix was added to the samples and incubated for 20-30 minutes, and then washed 3 consecutive times. Data acquisition was performed using non-fixed or fixed in 2%PFA buffer samples. All incubations

were performed on ice. Flow cytometry data were acquired with an LSR Fortessa flow cytometer (Becton Dickinson) and analyzed using FlowJo software (FlowJo LLC). Cell sorting was performed in a Sony SH800 Cell Sorter (Sony Biotechnology Inc.), the single (FSC-H versus FCS-W plot and SSC-H versus SSC-W) live (eFluor780^{-}) CD45^{+} cells were collected in RPMI medium with 2%FCS.

4.3.2 Mass cytometry (I-II)

eWAT and iBAT samples had to be pooled to obtain a sufficient number of leukocytes before staining. All incubations were performed at RT. The single cell suspensions were incubated in 2.5 µM Cell-ID Cisplatin for 5 min to exclude dead cells. The Fc-receptors blocking was done as for flow cytometry. The metal-tagged antibody mix was incubated for 30 minutes. Finally, the samples were incubated with DNA intercalation solution for 1 hour and fixed with 4% paraformaldehyde solution overnight at 4°C. Before the data acquisition in a Fluidigm Helios Mass Cytometer, the cells were washed and resuspended in purified H2O with 2mM EDTA. Data were analyzed using Cytobank software (Cytobank, Inc, I-III), VorteX clustering environment, and visualization of the resulting plots with Gephi 0.9.1 (https://github.com/nolanlab/vortex/releases/tag/29-Jun-2017, and https://gephi.org, I) or Cytosplore visual analysis system (Van Unen et al. 2017) (II).

Table 3. Murine antibodies used for cytometry (Flow–F & Mass–M) and histology (H).

ANTIBODY	SOURCE	CAT. #	RRID	F	M	H	PUB.
Viability dyes							
eFluor™ 780	eBioscience	65-0865		✓			I-III
Cisplatin 195Pt	Standard BioTools	201064			✓		I-II
Intercalator 103Rh	Standard BioTools	201103A			✓		I-II
Primary							
CD16/32	BioXCell	BE0307	AB_2736987	✓	✓		I-III
CD31	BD Biosciences	553370	AB_394816			✓	I; III
CD115	BioXCell	BE0213	AB_2687699		✓		I-III
CD144	BD Pharmigen	550548	AB_2244723			✓	I
CD163	BioRad	MCA342GA	AB_2074558	✓	✓		II
F4/80	BioRad	MCA497R	AB_323279			✓	I
RFP	Rockland	600-401-379	AB_2209751			✓	III
TIM4	BioLegend	130002	AB_1227802		✓		II-III

ANTIBODY	SOURCE	CAT. #	RRID	F	M	H	PUB.
Tyrosine Hydroxylase	Chemicon	AB152	AB_390204			✓	III
Tyrosine Hydroxylase	Chemicon	AB1542	AB_90755			✓	III
Perilipin-1	abcam	ab61682	AB_944751			✓	III
Fluorochrome conjugated							
aSMA-FITC	Sigma-Aldrich	F3777-2ML	AB_476977			✓	I
CD11b APC-Cy-7	BD Pharmingen	BD 557657	AB_396772	✓			I-II
CD11b BB515	BD Horizon	BD 564454	AB_2665392	✓			I
CD11b BV786	BD Pharmingen	BD 740861	AB_2740514	✓			I; III
CD11b PE	BD Pharmingen	BD 553311	AB_396680	✓			I
CD11c BV421	BD Horizon	BD 562782	AB_2737789	✓			I
CD11c BV605	BioLegend	117333	AB_11204262	✓			III
CD11c BV711	BioLegend	117349	AB_2563905	✓			II
CD31 APC	BioLegend	102509	AB_312916			✓	I
CD45 PerCP-Cy5,5	BD Pharmingen	BD 550994	AB_394003	✓			I-III
CD64 BV786	BD Pharmingen	BD 741024	AB_2740644	✓			II
CD64 PE	BioLegend	139304	AB_10612740	✓			I-III
CD68 FITC	BioLegend	137005	AB_10575475		✓		II
CD115 PE-Cy7	eBioscience	25-1152-80	AB_2573385	✓			I-III
CD117 A700	BioLegend	105846	AB_2783046	✓			III
CD117 BV510	BioLegend	135119	AB_2562011	✓			III
CD163 APC	BioLegend	155305	AB_2814059	✓			II-III
CD206 A488	BioRad	MCA2235A488T	AB_2297790	✓		✓	I-II
CD206 A647	BD Biosciences	565250	AB_2739133			✓	I
CD206 BV650	BioLegend	141723	AB_2562445	✓			I-II
CD206 PE/Dazzle	BioLegend	141731	AB_2565931	✓			III
CX3CR1 BV650	BioLegend	149033	AB_2565999	✓			II-III
F4/80 A488	eBioscience	53-4801-82	AB_469915	✓		✓	I-III
F4/80 A647	BioRad	MCA497A647	AB_323931	✓			I; III
F4/80 A700	BioRad	MCA497A700T	AB_1102556	✓			II
Ly6C BV421	BD Pharmingen	BD 562727	AB_2737748	✓			I-III
Ly6G BV510	BioLegend	127633	AB_2562937	✓			I-II
LYVE1 PE	R&D Systems	FAB2125P	AB_10889020		✓		II-III

ANTIBODY	SOURCE	CAT. #	RRID	F	M	H	PUB.
MerTK APC	R&D Systems	FAB5912A			✓		II-III
MerTK FITC	BioLegend	151503	AB_2617034		✓		II-III
MerTK PE	BioLegend	151505	AB_2617036	✓	✓		I
MHC II APC	eBioscience	17-5321-82	AB_469455	✓			II
MHC II PE-Cy7	eBioscience	25-5321-82	AB_10870792	✓			I-II
SiglecF A647	BD Biosciences	562680	AB_2687570			✓	I
SiglecF APC	BioLegend	155507	AB_2750236	✓	✓		II
SiglecF PE-CF594	BD Pharmingen	BD 562757	AB_2687994	✓	✓		I-III
TIM4 PE	BioLegend	130005	AB_1227807	✓			II
TIM4 PE Cy7	BioLegend	130010	AB_2565719	✓			III
Goat anti-Rabbit IgG (H+L) A488	Thermo Fisher Scientific	A-11034	AB_2576217			✓	III
Rabbit anti-Goat IgG (H+L) Recombinant A488	Thermo Fisher Scientific	A-27012	AB_2536077			✓	III
Goat Anti-Rat IgG (H+L) A488	Molecular Probes	A-11006	AB_141373			✓	III
Donkey Anti-Sheep IgG (H+L) A488	Molecular Probes	A-11015	AB_141362			✓	III
Metal conjugated							
B220 159Tb	Standard BioTools	3159015B			✓		I
B220 160Gd	Standard BioTools	3160012B			✓		II
CD4 172Yb	Standard BioTools	3172003B	AB_2811242		✓		I-II
CD8a 168Er	Standard BioTools	3168003B	AB_2811241		✓		I-II
CD11b 148Nd	Standard BioTools	3148003B	AB_2814738		✓		I-II
CD11c 142Nd	Standard BioTools	3142003B	AB_2814737		✓		I-II
CD19 166Er	Standard BioTools	3166015B	AB_2687846		✓		I-II
CD43 146Nd	Standard BioTools	3146009B			✓		II
CD45 89Y	Standard BioTools	3089005B	AB_2651152		✓		II
CD45 147Sm	Standard BioTools	3147003B	AB_2811243		✓		II
CD45 175Lu	Standard BioTools	3175010B			✓		I-II
CD64 151Eu	Standard BioTools	3151012B	AB_2814680		✓		I-II
CD80 171Yb	Standard BioTools	3171008B	AB_2885024		✓		I-II
CD115 144Nd	Standard BioTools	3144012B	AB_2895116		✓		II

ANTIBODY	SOURCE	CAT. #	RRID	F	M	H	PUB.
CD117 173Yb	Standard BioTools	3173004B	AB_2811230		✓		I-II
CD206 169Tm	Standard BioTools	3169021B	AB_2832249		✓		I-II
CD274 153Eu	Standard BioTools	3153016B	AB_2687837		✓		I-II
CX3CR1 164Dy	Standard BioTools	3164023B	AB_2832247		✓		I-II
F4/80 146Tb	Standard BioTools	3146008B	AB_2895117		✓		I
F4/80 159Tb	Standard BioTools	3159009B	AB_2811238		✓		II
Ly6C 150Nd	Standard BioTools	3150010B	AB_2895118		✓		II
Ly6C 162Dy	Standard BioTools	3162014B			✓		I-II
Ly6G 141Pr	Standard BioTools	3141008B	AB_2814678		✓		I-II
MHC II 174Yb	Standard BioTools	3174003B			✓		I-II
Siglec1 170Er	Standard BioTools	3170018B	AB_2885022		✓		I-II
TER-119 154Sm	Standard BioTools	3154005B			✓		II
TIM3 162Dy	Standard BioTools	3162029B	AB_2687841		✓		II
APC 176Yb	Standard BioTools	3176007B	AB_2811236		✓		II
FITC 144Nd	Standard BioTools	3144006B			✓		II
PE 165Ho	Standard BioTools	3165015B	AB_2714168		✓		I-II

4.4 Single-cell RNA sequencing (scRNAseq)(III)

We processed single live CD45+ cells sorted from the iBAT of animals at E17.5, P0, and P7 following the 10X Genomics guidelines (CG000126_Guidelines for Optimal Sample Prep Flow Chart RevA). To generate scRNAseq libraries, the Chromium Next GEM Single Cell 3′ Kit v3.1 (PN-1000268), Chromium Next GEM Chip G Single Cell Kit (PN-1000120), and Dual Index Kit TT Set A (PN-1000215), as instructed by the Chromium Next GEM Single Cell 3' Reagent Kit, Dual Index v3.1 User Guide (CG000315, Rev A) were used. The P0 BAT library was sequenced using Illumina's Hiseq3000 sequencer, while the E17.5 and P7 BAT libraries were sequenced using Illumina's Novaseq6000. The samples were sequenced to an average depth of approximately 50,000 reads/cell. Using the 10X Genomics Cell Ranger package (v2.0.0 or v2.1.1) demultiplexing, read alignment, and quality control were completed.

Using Seurat (ver 4.3), we performed graph-based clustering and differential gene expression analysis from the pre-processed data. Genes expressed in fewer than 3 cells, cells expressing less than 200 genes and cells with more than 5000 unique gene counts and 10% mitochondrial genes were filtered out. We used

"NormalizeData" and “FindVariableFeatures” functions to normalize the data, and to identify variable genes across single cells, respectively. We applied the ScaleData function to mitigate the impact of variability in UMIs, mitochondrial gene expression, and ribosomal gene expression between cells. Cells from the three samples were integrated, and FindClusters with a resolution of 0.8 was used for shared nearest neighbor graph-based clustering on the combined cells. The resulting clusters were visualized using Uniform Manifold Approximation and Projection (UMAP) through the RunUMAP and DimPlot functions.

Differentially expressed genes (DEG) between the clusters were calculated using the FindAllMarkers function in Seurat (ver 4.3). The parameters Min.pct (minimum percentage of gene-expressing cells in any cluster) and thresh.use (minimum fold change in gene expression between each cluster and all other clusters) were set to 0.25 (25%) and 0.69 (log2FC), respectively. We used the VlnPlot (Violin plots), DotPlot (dot plots), and DoHeatmap (heatmaps) functions for data visualization. Clusters exhibiting a macrophage signature were further subclustered with a resolution of 0.7, and DEG were analyzed as described above.

4.5 Bulk RNA sequencing (III)

Whole iBAT from P14 Plvap$^{-/-}$ mice and control littermates were collected and preserved in RNA*later*™ at -20 °C until RNA isolation. The Macherey-Nagel Nucleospin RNA kit was used for RNA isolation, eluted in RNase-free water, and stored at -70 °C. To ensure sample quality, Agilent Bioanalyzer 2100 was employed, and the concentration was measured using Qubit®/Quant-IT® Fluorometric Quantitation (Life Technologies) before and after library preparation. The library preparation step was conducted at the Finnish Functional Genomics Centre, University of Turku and Åbo Akademi, in collaboration with Biocenter Finland, using the Illumina Stranded mRNA Preparation, Ligation Kit (Illumina), following the Illumina Stranded mRNA Library Preparation protocol (1000000124518). Next-generation sequencing was carried out using the Illumina NovaSeq 6000 instrument.

For read alignment to the mouse genome build GRCm38, the HISAT2 alignment program (Mortazavi et al. 2008) was employed. Gene expression levels were estimated based on the abundance of transcripts (sequencing counts) mapped to the genome or exon. Read counts were normalized in relation to gene expression level, length, and sequencing depth. The FPKM (expected number of Fragments Per Kilobase of transcript sequence per Millions of base pairs sequenced) method was utilized to estimate gene expression levels (Mortazavi et al. 2008). Principal Component Analysis (PCA) was performed on the gene expression values (FPKM) of all samples after data filtering and normalization (CMP: counts per millions). Differentially expressed genes were then analyzed using the DESeq2 package

(version 1.36.0). Gene enrichment analyses of the differentially expressed genes were conducted using the Gene Ontology (GO) database (www.geneontology.org) and the Kyoto Encyclopedia of Genes and Genomes (KEGG).

Table 4. Bulk RNA iBAT.

ID	GROUP	[C] ng/µL	A260/A280	VOLUME (µL)
PV1_WT1	control	138,3	2,18	80
PV1_WT2	control	93,5	2,14	80
PV1_WT3	control	115,9	2,16	80
PV1_KO1	knockout	116,6	2,13	60
PV1_KO2	knockout	116,8	2,14	60
PV1_KO3	knockout	152,8	2,14	60

4.6 Quantitative polymerase chain reaction (I)

The total RNA of 5-week-old MG was isolated from sorted macrophages using the RNeasy Plus Micro kit. cDNA was obtained with a SensiFastTM cDNA Synthesis Kit (Bioline) according to the manufacturers' instructions. A quantitative polymerase chain reaction (qPCR) was performed using Taqman Gene Expression Assays (ThermoFisher Scientific) and acquired using either the QuantStudio12K Flex Real-Time PCR system (Thermo Fisher Scientific) or Quant Studio 3 Real-Time PCR System (Thermo Fisher Scientific). Using beta actin (Actb) as a control gene, relative expression levels were presented as % of Actb gene mRNA level from the same sample, using Applied Biosytems® analysis modules in the Thermo Fisher Cloud computing platform (ThermoFisher Scientific).

Table 5. The genes of interest and their qPCR probes.

GENE	**PROBE**
Actb	Mm02619580_g1
C1qa	Mm00432142_m1
Ccr2	Mm04207877_m
Csf1r	Mm01266652_m1
Cx3cr1	Mm00438354_m1

GENE	**PROBE**
Itgam	Mm00434455_m1
Itgax	Mm00498701_m1
Spi1	Mm00488142_m1
Zbtb46	Mm00511327_m1

4.7 Ductal branching of the MG (I)

The 4th branch of the MG of the specified mouse strains was collected whole and left to adhere briefly onto a glass slide and then fixed in Carnoy's fixative 4h at RT or overnight at 4ºC. Subsequently, samples were rehydrated through decreasing ethanol % series and stained with carmin alum following the manufacturer's protocol. Afterward, the samples were dehydrated and cleared in xylene for 2-3 days and mounted with Dibutylphthalate Polystyrene Xylene (DPX Mountant). The images were acquired with Axiovert M200 using a 5x/0.25 objective. Images were analyzed using 'Skeletonize2D/3D' and 'AnalyzeSkeleton' plugins in ImageJ by manually tracking and quantifying the area of the MG tree and a number of ductal branches.

4.8 Endocytosis assays

4.8.1 *In vivo* (I-II)

WT mice at 5 (I-II) or 22-week-old (II) were injected in the lateral saphenous vein with 0.8mg of 500 kDa dextran fluorescein (I-II), or 10 µg of fluorescently labeled acetylated low-density lipoprotein (I – LDL), or 5:1 immunocomplex (I-II – OVA-SIC). Immunocomplexes were pre prepared *in vitro*: 0.1 mg ovalbumin Atto488 (I) or fluorescein (II) + 0,02mg chicken egg albumin antibody complex incubated for 1 h at 4 °C(Stamatiades et al. 2016). PBS was injected as vehicle control.

After 1h (dextran) or 2h (LDL and OVA-SIC) incubation, MG and eWAT were collected, processed, and analyzed by flow cytometry (I-II) or immunofluorescence microscopy (I), as described above. For flow cytometry analysis, the mean fluorescence intensity (MFI) was obtained by subtracting the MFI of the control samples from the MFI of the treated samples MFI (I).

4.8.2 *In vitro* (II)

eWAT from 5-week-old mice was isolated and processed as described previously in the **Cytometry** section. SVF single cell suspension was diluted to 200 µl of RPMI-1640 supplemented with 10% FCS and 2 mM L-glutamine (Glutamax), and the cells were seeded to U-bottom 96-well plates. Samples were incubated with dextran-fluorescein or fluorescent beads (FluoSpheres carboxylate 0.5 µm) at +37°C for 1 h. After incubation, metabolic inactivation was performed by washing the samples with ice cold PBS. Negative control cells were kept at 4°C for 1 hour before being plated and exposure to the fluorescent cargoes was also performed at 4°C.

The antigen processing experiment was performed using DQ ovalbumin (DQ-OVA), as this substance only exhibits green fluorescence after proteolytic

degradation within the cells (Santambrogio et al. 1999). SVF was isolated from eWAT of 5- and 30-week-old diet challenged WT mice (see section 4.11) and incubated in the same conditions as formerly described.

Samples were acquired by flow cytometry. Phagocytosis/endocytosis and the proteolytic capacity of ATM subsets were calculated by dividing the MFI of the 37°C samples with the MFI of the 4°C samples.

Table 6. Substances used for the endocytosis assays.

SUBSTANCE		SOURCE	CAT. #	*In vivo*	*In vitro*	PUB.
Dextran 500 kDa	Fluorescein	Invitrogen	D7136	0.8mg	1mg/mL	I-II
LDL	A488	Thermo Fisher Scientific	L23380	10 µg	-	I
Ovalbumin	Atto488	Sigma	41235	5:1		I
Ovalbumin	Fluorescein	Invitrogen	O23020	5:1		II
Chicken egg albumin	-	Sigma	C6534	1:5		I-II
DQ-OVA		Invitrogen	D12053	-	10 µg/mL	
FluoSpheres carboxylate 0.5 µm	yellow-green 505/515	Invitrogen	F8813	-	1:1000*	

*0.002% of solids

4.9 Proliferation studies (I-II)

In vivo proliferation studies were performed by injecting i.p. 1.2mg of bromodeoxyuridine (BrdU), and tissue collection was performed after a 2h incubation for adults (I-II). Samples were processed as described above for flow cytometry; cells were then fixed and stained with an FITC Flow kit (I-II), according to manufacturers' instructions. Samples were acquired and analyzed as described for flow cytometry.

4.10 Histology

4.10.1 Formalin-fixed paraffin embedded (II-III)

The eWAT, the iBAT or the upper back were collected to PBS and fixed for 24 to 72h at RT with 10% normal buffered formalin. After fixation the samples were dehydrated in 50% to 70% EtOH gradient and kept in 70% EtOH at 4°C until paraffin embedding at the Histology core facility of the Institute of Biomedicine, University

of Turku, Finland. Samples were cut in 4µm sections onto glass slides, dried overnight at 37°C (II), deparaffinized, rehydrated, and stained with a Periodic Acid Schiff kit according to the manufacturer's instructions. Duplicates of the sections were stained with hematoxylin and eosin (not included in the publications). Slides were mounted with DPX Mountant, and left to dry in the fume hood for at least 48h. The samples were imaged using a Pannoramic 1000 (3DHISTECH) scanner (with a 40x lens) and analyzed using CaseViewer software (3DHISTECH – CaseCenter 2.9 SP1).

4.10.2 Frozen sections (I)

The MG was collected in PBS and embedded in the optimal cutting temperature compound (OCT), snap-frozen in liquid nitrogen, and sectioned (6-8µm) using a cryomicrotome (Leica). Before staining, the MG sections were fixed in ice cold acetone and blocked with 5% normal goat serum (NGS). Staining was performed at RT in a humidity chamber for 30min; see the antibody list in **Table 3**. The sections were mounted with ProLong Gold Antifade Mountant with DAPI, or the nuclei were stained with Hoechst and then mounted with ProLong Gold Antifade Mountant.

The images were acquired using a spinning disk confocal microscope (Intelligent Imaging Innovations) with a Plan-Apochromat 20 × /0.8 or 10 × /0.45 objective or LSM780 confocal microscope (Zeiss) with C-Apochromat 40 × /1.2 or C-Apochromat 63 × /1.2 oil objective. Image processing and analysis were performed with ImageJ software.

4.10.3 Whole-mounts (III)

The iBAT was collected in PBS, and cut with scissors into ≈0.3mm^2 pieces. Staining was done as previously described (Hofmann et al. 2021) with minor adjustments. The tissue was fixed overnight at 4°C in an IC (intracellular) fixation buffer. Blocking was performed with 1% species specific sera according to the antibody mix used (see **Table 8** and **Table 3**), 0.3% Triton X-100, and 1% FCS. Primary antibodies were incubated for 3 days at 37°C, secondary antibodies for 24-32 hours with one day wash with blocking buffer in between. After washing, in a washing buffer (PBS+0.2% Triton X-100 + 0.5% 1-thioglycerol), the samples were dehydrated at 4°C in isopropanol in Milli-Q H_2O gradient 30-100% for 3h and bleached for 2h (isopropanol + 5% DMSO + 2% H_2O_2). The samples were stored in a clearing medium (ECi: ethyl cinnamate) overnight before image acquisition. Image acquisition, processing and analysis were performed as before with a 3i CSU-W1 spinning disk confocal microscope EQUIPED WITH Photometrics Prime BSI

sCMOS camera and Plan-Apochromat Zeiss 10x/0.45 or 20x/0.8 dry objectives, using SlideBook 6 software and ImageJ software.

4.11 Dietary challenges (II)

Male mice from 7 to 8-weeks-old were weighted and distributed randomly into the different dietary groups fed *ad libitum* with: a natural ingredient-based standard diet [Chow; CRM (E), Special Diet Services, cat.: 801730, 3.62 kcal/g, 9.12 kcal% fat]; a purified ingredient normal-fat diet (NFD; 3.85 kcal/g, 10 kcal% fat); or a high-fat diet (HFD; 5.24 kcal/g, 60 kcal% fat) which induces obesity.

The main dietary challenges [Chow (n = 8), NFD (n = 8), and HFD (n = 16)] had a duration of 16 weeks. To study weight loss, two different dietary cohorts were started after 8 weeks on HFD: the metformin treatment group (Matsui et al. 2010) had metformin incorporated in the HFD food (HFD + Met, n = 8; 60 kcal% fat, average dose 353 mg/kg calculated based on food intake); and a reversed diet group that had the HFD changed to Chow diet (HFD to Chow, n = 10). Furthermore, eWAT was collected at 8 weeks in a specific diet (Chow & HFD) to set a baseline for the weight loss dietary cohorts (n = 8 for each group).

All mice from each dietary group were weighed every two weeks. After 8 and 16 weeks of a specific diet, quantitative nuclear magnetic resonance (NMR) scanning (EchoMRI-700, Echo Medical Systems) was used to assess the fat mass gain of the mice. Moreover, a glucose tolerance test (GGT) was performed after 14 weeks of diet intervention. Mice were fasted for 4h and injected i.p. with glucose (Glucosteril; 2.5 g/kg lean mass). Glycemia was measured with a glucometer (Contour XT, Bayer) from tail vein blood at 0, 20, 40, 60, and 90 minutes (II: Fig.4a).

Table 7. Diets used for the dietary challenges.

DIETS	SOURCE	CAT. #
Standard diet - Chow CRM (E)	Special diets Services	801730
High-fat diet	Research diets, Inc.	D12492
Normal-fat diet	Research diets, Inc.	D12450J
High-fat diet with Metformin	Research diets, Inc.	D18120701

4.12 Thermoneutrality studies (III)

Timed matings were set at RT, and the females' vaginal plug was checked every morning during the course of 5 days. If a vaginal plug was identified the female was separated from the male (E0.5). At E11.5±2days, the females were identified as

pregnant or not pregnant, and two separate cohorts were randomly created: pregnant female housed at RT vs pregnant female housed at 30ºC (Warming cabinet type 48-VSIII). The iBAT from the offspring of the two cohorts was collected at birth, 1 and 2 weeks, age matched but independently of sex. Samples were analyzed by flow cytometry.

4.13 Infrared thermography (III)

Plvap$^{+/-}$ x *Plvap*$^{+/-}$ offspring were randomly selected at 6 days of age. The pups were individually kept in a card box without a lid with separators in an area ranging from 49-92cm^2 for 30 minutes at RT (acclimatization) and then photographed with FLIR T620bx (infradex oy) thermal camera. Genotyping was performed around 10 days after birth, and the *Plvap*$^{-/-}$ and control littermates *Plvap*$^{+/+}$ were identified. The individual interscapular maximum temperature for each individual pup was analyzed using FLIR Thermal Studio – 1.9.40.0 software from FLIR systems.

4.14 Statistics (I-III)

Numeric data are given as mean ± SEM. GraphPad Prism (v6 and v9 GraphPad Software, LLC) was used for the statistical analyses. Comparisons between the time points, genotypes, or treatment groups were made using the nonparametric two-tailed Mann-Whitney test, a parametric two tailed t-test, and a one- or two- way ANOVA test with a Bonferroni post-hoc test. P-values lower than 0.5 were considered statistically significant.

Table 8. Reagents list.

	SOURCE	CAT. #	PUB.
Chemicals and Peptides			
10% normal buffered formalin	Sigma-Aldrich	HT501128-4L	II-III
1-Thioglycerol	Sigma-Aldrich	M1753	III
4% paraformaldehyde	Santa Cruz Biotechnology	SC-281692	I-III
Acetone	Merck	1.00014.2500	I; III
BrdU	BD Pharmingen™	51-2420KC	I-III
Carmin Alum	STEMCELL technologies	07070	I
Collagenase D	Roche	1108886601	I-III
Dibutylphthalate polystyrene xylene (DPX Mountant)	Sigma-Aldrich	06522-100ML	I-III
Dnase 1	Roche	10104159001	I-III

	SOURCE	CAT. #	PUB.
Dulbecco's phosphate buffered saline	Sigma-Aldrich	D8537	I-III
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich	D4540-100ML	II-III
EDTA 0.5M	Life Technologies	15575-038	II-III
Ethyl cinnamate (ECi)	Thermo Fisher Scientific	150531000	III
Fetal Calf Serum (FCS)	Biowest	S181B-500	I-III
Glucosteril 100 mg/ml	Baxter	-	II
Glutamax-I (100x)	Gibco	35050-038	II
Hanks' buffered saline	Sigma-Aldrich	H9394-500ML	I-III
Heparin 100 IU/mL	Leo	-	I-III
H_2O_2 solution, 30%	Sigma-Aldrich	H1009-100ML	
Hoechst 33342 Solution (20 mM)	Thermo Fisher Scientific	62249	
Intracellular (IC) Fixation Buffer	eBioscience	00-8222-49	III
Isopropanol	Merck	19516-500ML	III
Maxpar 10x barcode perm buffer	Fluidigm	201057	I-III
Maxpar fix and perm buffer	Fluidigm	201067	I-III
Normal Goat Serum (NGS)	Jackson ImmunoResearch	055-000-121	I; III
Optimal Cutting Temperature Compound (OCT)	Tissue-Tek	4583	I; III
PBS tablets	Gibco	18912-014	I-III
Percoll	Sigma-Aldrich	17-0891-01	I-III
Progesterone	Sigma-Aldrich	P0130-25G	I-III
Prolong Gold Antifade Mountant	Thermo Fisher Scientific	P36930	I
ProLong Gold Antifade Mountant with DNA stains DAPI	Thermo Fisher Scientific	P36941	I
RNA later	Qiagen	76106	III
RPMI-1640	Sigma-Aldrich	R5886-500mL	I-III
Sodium azide	Sigma-Aldrich	S2002-25G	I-III
Tamoxifen	Sigma-Aldrich	T5648-1G	I-III
Triton X-100	Sigma-Aldrich	T8787-250ML	I; III
UltraComp eBeads	Invitrogen™	01-2222-42	I-III
Xylene	VWR Chemicals	28973.363	I-III
Vectashield mounting media	Vector Laboratories	H-1000-10	III
Commercial kits			

	SOURCE	CAT. #	PUB.
Alexa Fluor 647 Protein Labeling Kit	Invitrogen	A20173	II
APC BrdU Flow kit	BD Pharmingen	552598	III
FITC BrdU Flow kit	BD Bioscience	559619	I-III
Foxp3 / Transcription Factor Staining Buffer Set	Invitrogen	00-5523-00	III
Maxpar® X8 Antibody Labeling Kit Tag-156Gd	Fluidigm	201156A	I-III
Maxpar® X8 Antibody Labeling Kit Tag-158Gd	Fluidigm	201158A	I-III
Maxpar® X8 Antibody Labeling Kit Tag-166Er	Fluidigm	201166A	I-III
Nucleospin RNA kit	Macherey-Nagel	740955.50	III
Periodic Acid Schiff Kit	Sigma-Aldrich	395B-1KT	II-III
RNeasy Plus Micro kit	Qiagen	74034	I
Vector® TrueVIEW® Autofluorescence Quenching Kit	Vector Laboratories	SP-8400-15	III

5 Results

5.1 Fetal-derived Macs in MG throughout development until adulthood

A previous study revealed that *Plvap*$^{-/-}$ mice had impaired MG ductal branching (Rantakari et al. 2016). As *Plvap*$^{-/-}$ mice have a defect in fetal liver-derived macrophages in most tissues we decided to investigate the ontogeny and function of murine MG tissue resident macrophages during development and adulthood in a steady state. Macrophages play a pivotal role in regulating (MG) morphogenesis (Chua et al. 2010; Gyorki et al. 2009; O'Brien et al. 2010), yet the precise characterization of the various subsets within this tissue remains incomplete. Thus, understanding the origins and functions of MG macrophages is essential for developing new therapies for diverse forms of mammary disorders.

5.1.1 Fetal-derived Macs dominate and persist in the MG

We started by assessing the presence of macrophages during the embryonic development of the 4th MG of female mice. Two main macrophage populations, defined by F4/80 expression were identified in the MG of E16.5 WT mice, as well as immediately after birth, suggesting the contribution of yolk sac and fetal liver to the macrophage pool in this tissue (I: Fig. 1a). When analyzing the MG from 1, 2, 5 and 12-13 -week-old WT mice, these two populations could still be identified, with F4/80^{Hi} macrophages being the dominant one (I: Fig. 1a, Sup. Fig. 2a and 2b). Interestingly, the same result was verified in 5-week-old 2nd, 3rd and 5th female MGs as well as for the 4th MG of male mice (I: Sup. Fig. 2e and f).

Fate mapping studies were used to explore the origin of these main macrophage populations in the MG. The *Cx3cr1*^{CreERT2}x*R26R-EYFP* (Yona et al. 2013) and *Csf1r*^{CreEsr}x*R26R-EYFP* (Qian et al. 2011) mouse models with a single tamoxifen pulse at E13.5 or E8.5, respectively, allow for the fluorescent labeling of yolk sac-derived macrophages/progenitors and their progeny. In newborn 2 and 5-week-old MG the YFP^{+} cells were mainly F4/80^{Hi} in both models (I: Fig. 1b, Sup. Fig. 2g), suggesting that yolk sac-derived macrophages contribute to the F4/80^{Hi} macrophage population and persist in 5 week-old murine MG. When yolk sac-derived

macrophages were selectively depleted with a single treatment of anti-CSF1R antibody at E6.5, the F4/80^{Hi} population was ablated but no effect in the F4/80^{Int} population at E17.5 was observed. However, in 2- and 5-week MGs the effects of the depletion were no longer detected (I: Fig. 1c). This result indicates that the yolk sac-derived macrophages in the MG do not constitute a major population in postnatal MGs.

The MG from the *Plvap*$^{-/-}$ mouse model at 3 and 5 weeks has a significant reduction in F4/80^{Hi} macrophages, while no significant change was observed in the F4/80^{Int} macrophage population. As the *Plvap*$^{-/-}$ mouse model has a reduction of fetal liver -derived macrophages but a normal contribution from YS and bone marrow in most tissues, these data suggest a major contribution of fetal liver -derived macrophages to the adult MG F4/80^{Hi} macrophage pool.

5.1.2 Monocyte-derived Macs in MG migrate CCR2-independently

CCR2$^{-/-}$ (Boring et al. 1997) and *Nur77*$^{-/-}$ (Lee et al. 1995) are mouse knockout models that lack specific blood Ly6C^{+} monocytes or Ly6C^{-} blood vessel patrolling monocytes, respectively. The frequencies of newborn *CCR2*$^{-/-}$ MG macrophage populations were comparable to the MG from the WT controls, demonstrating that fetal macrophage migration is CCR2 independent (I: Fig. 2a) (Hoeffel et al. 2015). At 2 and 5 weeks the *CCR2*$^{-/-}$ MG F4/80^{Int} population frequency was decreased when compared to the WT. This difference was attenuated in 3-month-old *CCR2*$^{-/-}$ MG (I: Fig. 2a). Conversely, the MG tissue resident macrophage population frequencies from *Nur77*$^{-/-}$ were comparable to the WT (I: Sup Fig. 3d), indicating that in a steady state blood vessel patrolling monocytes do not contribute to the MG resident macrophages.

MG macrophage and circulating monocyte depletion was achieved in 3 rounds of consecutive injections of anti-CSF1 antibody and clodronate liposomes (I: Sup. Fig. 3g). After an 11-day recovery, the F4/80^{Int} macrophage population was restored in the MG, but F4/80^{Hi} macrophages were still undetectable (I: Fig. 2d), which proves the contribution of bone marrow-derived macrophages to the F4/80^{Int} macrophage population, but not to the F4/80^{Hi} population.

Together, these results show that bone marrow-derived monocytes significantly contribute to the F4/80^{Int} tissue resident macrophage population in the postnatal MG, and the influx of these cells is mainly done in a CCR2-independent manner, as opposed to what occurs in primary mammary tumors (Qian et al. 2011).

5.1.3 Fetal-derived Macs in MG are defined by CD206 expression

High dimensional mass cytometry analysis demonstrated that not only the F4/80 membrane marker characterizes the tissue resident macrophage populations in the MGs at 5 weeks and 3-months-old old as this can also be demonstrated by CD206. Interestingly, the expression of CD206 separates F4/80 macrophages into two distinct populations: the $CD206^{Hi}$ population that also expresses high levels of F4/80, and other canonical macrophage markers, such as CD64, Siglec-1 and MerTK and the $CD206^{Neg/low}$ macrophages that express lower levels of F4/80 but higher levels of CX3CR1 and MHC II, which are also expressed by monocytes (I: Fig3a, c and d). When implementing an unsupervised clustering algorithm, X-shift, for the visualization of the predictions of the cell-fate and cell relations according to the leukocyte markers expressed, the hierarchical development of $CD206^{Neg/low}$ suggested the evolution of this population from different bone marrow-derived monocytes (I: Fig3e and Sup. Fig.6c).

Furthermore, flow cytometry data from the MG of 5-week-old WT mice showed that the $CD206^{Hi}$ macrophages also express $F4/80^{Hi}$ and high levels of macrophage-specific transcripts (I: Fig.4a and b). Yolk sac-derived CX3CR1-YFP-labeled macrophages only contribute to the $CD206^{Hi}$ macrophage population in MGs from 5-week-old reporter mice (I: Fig.4c). Interestingly, this same population was partially ablated in the MG from the 5-week-old *Plvap*$^{-/-}$ mouse model (I: Fig.4d). It was not recovered after macrophage depletion treatment (I: Fig.4f). These data support the notion that fetal-derived macrophages in MG are defined by CD206 expression.

5.1.4 CD206 Macs are vital for MG development and function

Notably, carmine alumine whole-mount staining of 5-week-old MGs from different genotypes implied that fetal liver -derived macrophages are necessary for MG branching morphogenesis, as only the *Plvap*$^{-/-}$ mouse model MG showed impaired branching (I: Fig.5a). Additionally, fetal-derived $CD206^{Hi}$ macrophages were shown to have a greater scavenger function compared to bone marrow-derived $CD206^{Neg/low}$ when analyzing MGs from 5-week-old mice injected with fluorescently labeled particles (dextran, acetylated LDL and immunocomplexes) (I: Fig.5b). Frozen section immunofluorescent staining showed that $CD206^{+}$ macrophages are co-localized with vessels and ducts, which is in line with their scavenger function, as seen in I: Fig.5f, where a dextran particle has been phagocytosed by a $CD206^{+}$ macrophage that co-localizes with a blood vessel.

Thus, MG fetal-derived CD206^{Hi} macrophages are vital for the development of the gland and constitute the preferential blood-borne ligands scavenger population in the MG.

5.2 Heterogeneity of resident Macs in white adipose tissue (WAT)

The MG is based on a fat pad, and the tissue resident macrophages are in contact with adipocytes (Howard and Lu 2014). As MG macrophages originate from the fetal liver and significantly impact tissue development and function, we aimed to investigate the macrophage ontogeny and functionality within WAT.

5.2.1 WAT-resident Macs are defined by the expression of CD206, TIM4, and CD163

Mass cytometry analysis of steady state eWAT at 2, 5, 8, 12, and 23-weeks-old WT mice revealed a complex landscape of different CD45$^+$immune cells, (II: Sup. Fig.1c). Further analysis of the tissue resident macrophages, CD45$^+$ CD11b$^+$ CD64$^+$ F4/80$^+$, confirmed that the majority of subpopulations express CD206, with only a fraction lacking the CD206 expression which are, most likely, monocyte-derived cell populations (II: Fig.1a). CD206 is a canonical macrophage marker associated with an anti-inflammatory macrophage phenotype also known as M2-like macrophage (Mills et al. 2000; Sica and Mantovani 2012; Sindrilaru et al. 2011).

A FlowSOM (Van Gassen et al. 2015) algorithm performed unsupervised clustering of the tissue resident macrophages according to the different surface cell markers expressed (II: Fig.1b). Subsequently, four primary subpopulations were identified based on TIM4 and CD163 expression, henceforth referred to as TIM4$^+$CD163$^+$, TIM4$^+$(CD163$^-$), (TIM4$^-$)CD163$^+$ and TIM4$^-$CD163$^-$. Each one of these four subsets could be further subdivided according to MHC II expression, as positive or negative. When focusing on the kinetics of the tissue resident macrophages present in eWAT from juvenile (2 and 5-week-old) and adult (8, 12, and 23-week-old) mice, it became clear that the MHC II$^+$ subpopulation fraction was becoming predominant in each of the four main populations during aging. Additionally, VAMs, thought to be the main macrophage population in lean WAT (Silva et al. 2019), were identified based on the cell markers expressed in all the 12 subpopulations (II: Sup.Fig. 4). The collected data has helped to enhance the comprehension of the macrophage heterogeneity present in lean WAT, and revealed a greater complexity in the subtypes of adipose tissue resident macrophages in a steady state.

5.2.2 The ontogeny of ATM subpopulations

To understand the contribution of bone marrow-derived cells to the resident macrophages in lean adipose tissue, mass cytometry was used to analyze 5 and 8-week-old murine eWAT of *CCR2*$^{-/-}$ (Boring et al. 1997) and *Nur77*$^{-/-}$ (Lee et al. 1995) knockout models, which are deficient in Ly6C^{+} monocytes or Ly6C^{-} blood vessel patrolling monocytes, respectively. The frequency of CD163^{+}MHC II^{+} and TIM4^{-}CD163^{-}MHC II^{+} subpopulations significantly decreased in *CCR2*$^{-/-}$ eWAT, suggesting their bone marrow origin and CCR2 dependence (II: Fig.2d, g, and h).

Fetal-derived macrophages from the yolk sac and/or fetal liver, are still present in most tissues after birth and throughout life. Three different cell-fate mapping experiments were conducted to understand the fate of yolk sac -derived cells in eWAT (see 4.1.1). Flow cytometry analysis of the 5-week-old reporter eWAT revealed that the labeled YFP^{+} cells fell preferentially into the TIM4^{+}CD163^{+} subpopulation (II: Fig.3b, d and f and Sup. Fig. 6b), and that there is a significant decrease in the number of YFP^{+} cells at 10 weeks of age eWAT (II: Sup. Fig.6c), implying that TIM4^{+}CD163^{+} subpopulation is yolk sac-derived and that these cells have a limited lifespan in aging mice. To assess whether the adipose tissue resident macrophage populations were self-maintaining due to local proliferation the dividing cells were quantified *in vivo* using BrdU labeling. TIM4^{+}CD163^{+} and CD11c^{+} subpopulations predominantly incorporated the synthetic nucleoside at 5 and 16 weeks, and that at 16 weeks the CD11c^{+} population had a higher rate of cell division (II: Fig.3g), demonstrating that only these two populations are self-renewing *in situ*. Furthermore, the absence of BrdU incorporation by CD163^{+} and TIM4^{-}CD163^{-} subpopulations supports the bone marrow origin hypothesis, as their replenishment is possible through CCR2-dependent monocytic recruitment.

5.2.3 The major WAT-resident macrophage subpopulations persist through metabolic challenges

Different dietary challenges were introduced to the WT mice (see 4.11, II: Fig.4a) – Chow, NFD, HFD, HFD + Met and HFD to Chow – to understand if obesity or weight loss influences the ATM immune profile. Using HSNE (Pezzotti et al. 2016; Van Unen et al. 2017) (hierarchical stochastic neighboring embedding) clustering of the CyTOF data, the same steady-state CD206^{+} populations were identified in all the diet groups, except for TIM4^{-}CD163^{-} that was merged with the CD206^{-} inflammatory population on HFD and HFD+Met samples (II: Fig.4b, c and d).

The cell counts, emphasized by higher resolution embeddings of the different populations, revealed that TIM4^{+}CD163^{+}, TIM4^{+}, and CD163^{+} kept their numbers steady, even when a significant influx of inflammatory cells was observed in the TIM4^{-}CD163^{-} & CD206^{-} subpopulations of the HFD and HFD+Met groups, (II:

Sup.Fig.8b). Interestingly, this influx of inflammatory cells regressed when the animals were fed a Chow diet after obesity is induced (HFD to Chow). Moreover, an MHC II phenotypic switch could be observed when obesity was induced, as the HFD and the HFD + Met groups displayed downregulation of MHC II when compared to the Chow, NFD, and HFD to Chow groups.

To further understand this MHC II phenotypic switch, the CyTOF macrophage ($F4/80^{+}CD64^{+}$) data was analyzed under an unsupervised FlowSOM algorithm for better quantification. Once again, the main 4 subpopulations were identified, with the addition of the expected $CD206^{-}CD11c^{+}$ populations, mainly identified in eWAT from HFD and HFD + Met (II: Fig5a and Sup.Fig9b). MHC II expression within the main 4 populations was studied in detail (II: Fig.5b-e) and a majority of MHC II^{+} cells in all 4 populations of Chow and NFD groups were unraveled. In contrast, when obesity is induced with HFD, there is a clear MHC II phenotype switch primarily in the embryonic-derived $TIM4^{+}CD163^{+}$ and $TIM4^{+}$ populations, which is already evident at 8 weeks in HFD (II: Sup.Fig. 9d). Notably, this phenotype is rescued to a steady-state when the HFD is substituted by Chow, but not after metformin treatment. These data suggest a macrophage origin-dependent MHC II response to diet-induced obesity.

5.2.4 Functionality of WAT macrophage subtypes

All 4 main populations of ATM in 5-week-old mice eWAT showed an endocytic capacity of dextran or fluorescent beads *in vitro* (II: Fig.6b and Sup. Fig10b), acting as genuine macrophages with a phagocytic function. Dextran was preferentially phagocytosed by embryonic-derived $TIM4^{+}CD163^{+}$ as opposed to $TIM4^{-}CD163^{-}$ (II: Fig.6b and Sup. Fig10c). MHC II^{+} clusters were more efficient in phagocytosis in $TIM4^{+}$, $CD163^{+}$, and $TIM4^{-}CD163^{-}$populations. Apart from being able to phagocytose particles, ATM populations also exhibited proteolytic function. Notably, $TIM4^{+}CD163^{+}$ degraded DQ-OVA above $TIM4^{-}CD163^{-}$ and $CD163^{+}$ populations (II: Fig.6c and Sup. Fig. 10e). The SVF of eWAT from 23-week-old mice under dietary challenges exhibited the higher proteolytic capacity of $CD163^{+}$ and $TIM4^{-}CD163^{-}$populations (II: Chow group Sup. Fig.10g) as opposed to what was observed at 5 weeks. Ultimately, all 4 main macrophage populations can process soluble antigens, and this function is altered with age but not by different diets, *in vitro*.

To investigate the phagocytic capacity of the different 4 populations of ATM *in vivo*, 5 and 22-week-old mice were injected (i.v.) with fluorescently labeled dextran and 5-week-old mice with OVA-SIC (II: Fig.6d and Sup. Fig.10j). Overall, $TIM4^{+}CD163^{+}$ and $CD163^{+}$ were more efficient in phagocytosing dextran from the blood at 5 weeks, nevertheless, the $TIM4^{+}CD163^{+}$ population had superior

scavenging function. Interestingly, with age, TIM4^{+}CD163^{+} had a significant reduction of dextran uptake, indicating, again, that age influences macrophage function. OVA-SIC was primarily processed by TIM4^{+}CD163^{+}, even though eWAT is not a preferential site for immunocomplexes up-take (II: Sup. Fig.j). In conclusion, embryonic-derived TIM4^{+}CD163^{+} macrophage population has a superior scavenging capacity.

5.3 Pre and early postnatal brown adipose tissue (iBAT) Macs

iBAT has emerged as a promising target for preventing and treating obesity (Cheng et al. 2021; Poher et al. 2015; Raiko et al. 2020; Wibmer et al. 2021). Upon discovering a stable population of embryonic-derived macrophages in WAT, we began to question whether a similar phenomenon existed in iBAT and, if so, what role these macrophages played in the cross-talk between β-adrenergic fibers and brown adipocytes essential for this tissue function.

5.3.1 Macrophages populate iBAT before birth

Our flow cytometry analysis of pre (E15.5, E17.5) and postnatal day (P) 0 murine iBAT confirmed the existence of macrophages during the embryonic development of the tissue (III: Fig. 1a). We identified two F4/80 myeloid populations, F4/80^{Int} and F4/80^{Hi}. However, iBAT F4/80^{Hi} population is almost non-existent, constituting less than 10% of myeloid cells (III: Sup. Fig.1b), hinting at a virtual lack of contribution from yolk-sac derived cells to the macrophage resident pool in iBAT. We further collected juvenile (P7 and P14) and adult (P56 – 8 weeks of age) iBAT samples, and verified that F4/80^{Hi} macrophage population was still missing (III: Fig.1b and Sup. Fig. 1b). The total number of macrophages increases alongside the growth of the tissue peaking at 1-week-old, and then decreases throughout adulthood (III: Fig.1c).

As in adult iBAT CX3CR1^{+} macrophages control tissue innervation (Wolf et al. 2017), therefore we analyzed the main macrophage population (F4/80^{Int}) for differential expression of several membrane cell markers (III: Fig. 1d and e). The expression of CX3CR1 remains steady throughout all time points, even with a high increase in the number of CX3CR1^{+} cells at 1 week and the subsequent decrease throughout aging. Interestingly, Ly6C^{+} and CX3CR1^{+} macrophage numbers remained quite similar at all time points. Overall, these data prove the existence of macrophages already during embryonic development of iBAT and that the majority of the macrophages have a monocytic progenitor.

We performed scRNA-seq on WT iBAT leukocytes from E17.5, P0, and P7. The unsupervised clustering demonstrated a complex heterogeneity of different

leukocytes residing in the developing iBAT (III: Sup. Fig. 1g), the macrophage cluster being the dominant one. We subclustered the macrophages according to the expression of *Adgre1* (codes F4/80), *Fcgcr* (codes CD64) and *Csf1r* (codes CSF1R/CD115), as they these transcripts are well-characterized murine macrophage markers (Byrne, Guilbert, and Stanley 1981; Gautier et al. 2012; Waddell et al. 2018). At all time points studied we found 9 macrophage clusters, with most clusters maintaining a monocytic signature by expressing *Ly6c* and *Ccr2* (III: Fig. 1f-g and Sup. Fig. 1j-k), which supports our flow cytometry findings. Each macrophage cluster had a specific transcriptomic signature, for example clusters 1 and 2 exhibit a Ly6C^{Hi} and Ly6C^{Low} signature, respectively; cluster 3 was enriched with genes related to activated microglia; and cluster 4 shared identity with vascular-associated macrophages (III: Sup. Table 1) according to the expressed genes.

These results elucidate the complex macrophage landscape already existing during iBAT's embryogenesis, suggesting that macrophages may be necessary for these stages of development.

5.3.2 Fetal liver-derived macrophages are the main population in developing iBAT

To understand the origin of the early stage iBAT resident macrophages, we employed different cell-fate mapping strategies. Firstly, we investigated the fetal liver contribution to the iBAT resident macrophage pool. We used *Ccr2*^{CreERT2}x*TdTomato*^{lox} mice (Croxford et al. 2015), and induced TdTomato labeling with two tamoxifen injections, one at E14.5 and other at E16.5. We tracked the TdTomato^{+} cells at E17.5 and P14. Flow cytometry analysis revealed that at E17.5 around 80% of the total TdTomato^{+} myeloid leukocytes fall into the F4/80^{Int} macrophage population. However, at P14, the frequency of TdTomato^{+} cells was already diminished (III: Fig. 2b). We then addressed the contribution of the yolk sac-derived precursors using *Cx3cr1*^{CreERT2}x*R26R-EYFP* model (Yona et al. 2013). We gave the tamoxifen pulse at E9.5, and we tracked the YFP^{+} cells at E17.5, finding only a negligible amount of YFP^{+} cells (III: Sup. Fig. 2e). Lastly, we used the *Ms4a3*^{CreERT2}x*TdTomato*^{lox} model (Liu et al. 2019) with a single tamoxifen pulse at P5, targeting bone marrow-derived cells. At P7, more than 70% of the total TdTomato^{+} myeloid leukocytes fall into the F4/80^{Int} macrophage population, signifying that bone marrow-derived macrophages are already infiltrating iBAT (III: Fig. 2d). Here we prove that fetal liver precursors constitute the main population of macrophages present during early stage iBAT development, without yolk sac progenitor contribution. Additionally, the turnover of bone marrow-derived macrophages already occurs at 2 weeks of age.

5.3.3 iBAT fetal liver-derived macrophages interact with developing neuronal network

Remarkably, at E17.5 we found that the TdTomato^{+} macrophages (fetal liver-derived) organize in clusters within the tissue stroma and are strictly associated with TH fibers (III: Fig. 2e). This co-localization with the β-adrenergic fibers suggests a possible involvement of macrophages and iBAT function already at this stage of development. To further understand the contribution of fetal derived-macrophages to iBAT function, we used *Plvap*$^{-/-}$ mouse model that lacks fetal liver-derived macrophages in most tissues (Rantakari et al. 2016).

Flow cytometric analysis confirmed a significant reduction in the F4/80^{Int} population but not in the F4/80^{Hi} (III: Fig. 3a) at E17.5. At 1 week this reduction in F4/80^{Int} is no longer identifiable, most likely because of infiltration of bone marrow-derived macrophages already at 1 week of age (III: Sup. Fig.3a). Histological analysis showed delay in *Plvap*$^{-/-}$ iBAT development during embryonic development. At E17.5 the KO brown adipocytes islands are not merged, being more similar to E15.5 WT iBAT (III: Fig. 3b). However, at one week *Plvap*$^{-/-}$ iBAT's structure is already comparable to WT (III: Sup. Fig. 3c). Interestingly, the *Plvap*$^{-/-}$ sympathetic nervous network structure is comparable to that of the WT littermates (III: Fig. 3c).

We performed iBAT bulk RNA analysis from P14 *Plvap*$^{-/-}$ animals, taking advantage of the tissue's activity peak described at this time point (Liu et al. 2020). Analysis of differentially expressed genes (DEG) showed that from 12614 genes, 792 are *Plvap*$^{-/-}$ specific and 420 WT specific (III: Fig. 4a). Additionally, there was downregulation of genes related to, for example, adipogenesis (e.g. *Col6a2*, *Hrasls*, *Gdf6*, *Nxn*) and neuronal activity (e.g. *Mbp*, *Matn2*, *Prx*) (III: Fig. 4c). To understand the biological value of the differentially expressed genes we performed pathway enrichment analysis and found that many of downregulated genes are involved in thermogenesis, PPAR signaling, and insulin secretion pathways (III: Fig. 4d). These results suggest that fetal liver-derived macrophages may be involved in iBAT development and function.

As the adaptation to the extra-uterine cold environment after birth is mainly achieved by non-shivering thermogenesis (Cannon and Nedergaard 2004), we employed infrared thermography to quantify the iBAT temperature. P5 *Plvap*$^{-/-}$ animals had a lower body temperature when compared to WT littermates (III: Fig. 4f). Taken together, these results propose that a deficiency in fetal liver-derived macrophages might interfere with BAT function.

6 Discussion

Embryonic macrophages colonize the tissues during development and persist throughout adulthood (Hoe and Ginhoux 2018; Hoeffel and Ginhoux 2015). These tissue resident macrophages acquire functions specific to the tissue they populate and readily respond to environmental changes. Understanding the origin and functions of these specialized macrophages might help find potential targets to prevent or treat specific diseases.

We focused our study first on the MG, as previous findings in our lab lead us to believe that embryonic-derived macrophages are the main population in this organ even during adulthood (Rantakari et al. 2016). The stroma of the developing MG is mainly constituted by adipocytes, and mammary macrophages are in close contact not only with the gland's epithelia but also with the adipocytes (Stewart et al. 2019). The adipose organ is spread around the whole body, forming different kinds of fat pads. Thus, we decided to examine if macrophages are also involved in the development of the different pads. In order to do this we studied the eWAT and the iBAT at different developmental phases.

6.1 Fetal liver-derived macrophages dominate in adult MGs

The major development of the MG is triggered at the onset of puberty, and during adulthood, the different phases of a female's hormonal cycle highly influence the gland's regeneration. Every stage of development has an effect on the MG stroma environment, including the immune cells. Macrophages have been described as playing a key role in the healthy development of the gland, specifically during the morphogenesis of the branches. Even though the MG only fully develops after birth, the initial development of this tissue already starts around E10 (Cowin and Wysolmerski 2010).

6.1.1 Macrophages seed the MG during embryonic development and persist throughout postnatal life

At the time we published the **Publication I**, it was thought that macrophages only populated the murine MG at 2 weeks of age, surrounding the nipple area and the undeveloped ductal structures (Gouon-Evans et al. 2000). However, we studied the 4th MG from females at E16.5 and identified two subsets of macrophages already at this stage. These subsets were putatively distinguished as the fetal liver and yolk sac-derived by their F4/80^{Int} and F4/80^{Hi} expression, respectively (Ginhoux and Guilliams 2016; Hashimoto et al. 2013; Perdiguero and Geissmann 2015; Schulz et al. 2012; Varol et al. 2015; Yona et al. 2013). We traced the macrophage populations throughout development and the F4/80^{Hi} population dominated during pre and postnatal life. Notwithstanding, at birth and 2-weeks-old, the F4/80^{Int} population had a similar frequency as the F4/80^{Hi} population. It is important to note that at 2 weeks of age, the bone marrow is already contributing with monocyte-derived macrophages to the resident pool of F4/80^{Int} macrophages in several tissues (Shevyrev et al. 2023). This first wave of bone marrow seeding, and the gradual enrichment of F4/80 expression of the fetal liver-derived macrophages after birth might be the reasons why at this time point both F4/80 populations are balanced.

We also collected the 2nd, 3rd and 5th MGs from females at 5 weeks of age, coinciding with the puberty period and when MG triggers its main development after birth. At 5 weeks, all the MGs resembled each other when comparing the macrophage subsets frequencies, suggesting a conserved function for macrophages in MG development. Additionally, the 4th MG of 5-week-old males was also collected and the macrophage populations were analyzed. We found that the macrophage subsets also resembled the ones present in females. Interestingly, in male mice MG degenerates during the embryonic period, around day E13.5-E15.5 (McNally and Stein 2017). At this stage, macrophages have been identified in direct contact with the mammary bud, probably phagocyting apoptotic cells (Stewart et al. 2019). Therefore, the presence of macrophages in the 5-week-old 4th MG of male mice might not be related to the development of the gland but to adipose tissue function, as in male mice the MG adipose tissue is known as the subcutaneous fat pad. Nevertheless, it has been verified that perinatal contact with estrogen-like substances might trigger postnatal male gynecomastia - MG development (Szabo and Vandenberg 2021; Vandenberg et al. 2013). Perhaps, the macrophages existing in the male MG may promptly support this developmental surge.

The use of reporter mice and the depletion of macrophages at E6.5 confirmed the existence of yolk sac-derived macrophages in the MG and their self-maintenance postnatally at 5 weeks of age. However, yolk sac-derived macrophages were not identified as a major population in the postnatal MG. We then checked MG from *Plvap*$^{-/-}$ mice that have a reduction of fetal liver-derived macrophages in most tissues

(Rantakari et al. 2016). A significant reduction of the F4/80^{Hi} population was described at 3 and 5 weeks of age, with no effect on the F4/80^{Int} macrophage subset. These data are in agreement with the studies describing the fetal liver as the main hematopoietic organ during embryonic development and the major supplier of macrophages to most tissues (Lewis et al. 2021; Sheng et al. 2015).

6.1.2 MG's bone marrow-derived macrophages are mainly CCR2-independent

Although we found that fetal liver-derived macrophages seed the main macrophage subset present in a steady state MG of virgin females, it is essential to understand the kinetics of all different tissue resident macrophage subsets. The resident macrophages in the MG can influence the tumorigenesis of breast cancer (Hirano et al. 2023). CCL2 and CSF1 were shown to be essential for the recruitment and polarization of bone marrow-derived macrophages in breast cancer (Yang et al. 2022). Nevertheless, in our experiments only a minority of the bone marrow-derived macrophages were identified as CCR2-dependent, as at 2 weeks, 5 weeks and 3 months of age only a small portion of F4/80^{Int} was affected by the absence of CCR2. At birth the *CCR2*$^{-/-}$ mice exhibited the expected frequencies of both macrophages subsets, F4/80^{Int} and F4/80^{Hi}, since fetal monocytes can express CCR2 but are not dependent on CCL2 for migration (Hoeffel et al. 2015). These data prove that steady state conditions are very different from that seen in the case of disease. Even though CCR2-dependent bone marrow-macrophages are not a main subset in steady state MG, these cells have a significant contribution to the progression of neoplasia (Kersten et al. 2017). Moreover, the depletion of the MG resident macrophage populations showed that the F4/80^{Hi} subset is not readily replenished by the bone marrow. However, with a longer recovery time populations of bone marrow-derived macrophages might start to repopulate and resemble the depleted subset, as seen in multiple other tissues (Epelman et al. 2014; Guilliams et al. 2020).

6.1.3 CD206 defines the embryonic-derived macrophage populations

Remarkably, our data identified CD206 as a good marker for embryonic-derived macrophages. In *Plvap*$^{-/-}$ mice, the CD206^{+} population was decreased, and in depletion experiments this population was also not recovered. These embryonic CD206^{+} macrophages were found to be closely associated with the vessels and to be highly endocytic, indicative of an immune surveillance function. Moreover, CD206^{+} macrophages were also localized in close contact with MG ducts, suggesting a possible involvement in the remodeling of the ducts. In the MG of *Csfm*^{op}*/Csfm*^{op}

mice (lacking CSF1), there is a partial depletion of macrophages around the terminal end bud, resulting in delayed ductal expansion. However, the network is eventually fully developed by 12 weeks of age (Gouon-Evans et al. 2000). Our *Plvap*$^{-/-}$ mice still show the absence of ductal growth at 5 weeks. As fetal liver-derived macrophages are not CSF1R-dependent (Hoeffel et al. 2015; Squarzoni et al. 2014) they are possibly the remaining macrophage population found in *Csfm*^{op}/*Csfm*^{op} mice MGs, and ultimately, the macrophages responsible for the development of the ductal network in this mouse model, albeit a late one.

The MG is an organ highly responsive to ovarian hormones, and in our study, unfortunately, we did not check in which estrous cycle phase the females were in before tissue collection. It has been described that MG macrophage populations fluctuate according to the female cycle, accompanying the growth and regression stages of the gland (Chua et al. 2010; Hodson et al. 2013). However, the macrophage subsets that we analyzed were not especially variable. This might be associated with the fact that we cage our females in social groups without a male, triggering a suppression of the estrus – Lee-Boot effect (Champlin 1971), which decreases the variability of the cycles in mature females. Additionally, our *Plvap*$^{-/-}$ mice do not surviving longer than 5 weeks (Stan et al. 2012), which did not allow us to study the influence of fetal liver-derived macrophage deficiency in the sex hormone-cycles of the mice.

6.2 Heterogeneity of the WAT macrophage landscape

Overweight and obesity have emerged as major concerns in human health due to their association with an elevated risk of developing a range of chronic conditions and diseases. White adipocytes expand in size and number to allocate the fat meant for storage. When too much fat is available, the growth of WAT is exponential and imbalanced. Macrophages are the main immune cell present within this tissue and key players in the development of the chronic state of low-grade inflammation associated with obesity. The influx of bone marrow-derived macrophages in the context of obesity has been widely studied. However, the first macrophages to proliferate and respond to an imbalanced growth of the WAT already reside within the tissue in a steady state (Amano et al. 2014; Zheng et al. 2016). We focused on the study of the ontogeny of these adipose tissue resident macrophages (ATMs) and their role in homeostatic or obesity-related functions.

6.2.1 Existence of eight CD206$^+$ main populations

Using CyTOF, we were able to apply a broader panel of membrane cell markers, in an attempt to unify the knowledge of the main subsets of ATM already described in the literature review. As WAT fat pads start to expand after birth, we collected eWAT starting from animals at 2-weeks-old until 23-week-old. We verified that in a steady state, most ATM populations are CD206$^+$, according to the previously described ATM subsets (Nawaz et al. 2017; Nawaz and Tobe 2019). Thus, in contrast to what was established in the MG, CD206 is not a good marker to describe embryonic origin macrophages in WAT, as it is expressed by most macrophage populations in postnatal animals. We also found that the differential expression of TIM4 and CD163 defined four different subsets: TIM4$^+$CD163$^+$, TIM4$^+$(CD163$^-$), (TIM4$^-$)CD163$^+$, and TIM4$^-$CD163$^-$. Within each subset, we identified an MHC II$^+$ and MHC II$^-$ population, with the positive population increasing with age.

WAT is a highly vascularized organ supporting the transport of several growth factors, cytokines, and hormones (Herold and Kalucka 2021). Some ATM subsets have been described to be in close contact with the vessels, having a high endocytic capacity, and constituting the main subsets of ATMs in a steady state. These vascular-associated macrophages (VAMs) were not readily replenished by the bone marrow, suggesting an embryonic origin (Silva et al. 2019). However, another macrophage population expressing CD206 and Lyve1, has also been established as a blood vessel invested in several tissues, including adipose tissue, but defined as bone marrow-derived (Chakarov et al. 2019; Lim et al. 2018). We investigated if any of our 8 main populations would overlap with these previously described vascular macrophages. Interestingly, none of the subsets coincided exclusively with the VAMs or Lyve1$^+$ macrophages existing within each one of our main populations. This suggests that all the 8 subsets described by us have the characteristics of macrophages associated with the vessels, and that being associated with vessels does not define ATMs. Another possibility is that ATMs are generally close to the vessels, as eWAT is a highly vascularized tissue (Cao 2007).

Interestingly, tissue resident macrophages in the omentum, a different visceral fat pad, have already been similarly defined according to TIM4 and CD163 expression (Etzerodt et al. 2020). Moreover, these populations were all CD169 (also known as Siglec1) positive, which also coincides with our results from CyTOF (see II: Sup.Fig.2). These data strengthen our macrophage subtyping. Notably, all the omentum tissue resident macrophages subsets were also positive to Lyve1, suggesting that, even though some similarities may be shared across fat pads, not all characteristics can be generalized.

6.2.2 Ontogeny of ATM

The *CCR2*$^{-/-}$ mouse model has been widely studied in the context of obesity, as the influx of macrophages associated with this disease is mainly CCR2-dependent. However, the contribution of CCR2-dependent monocytes to the steady state pool of ATMs has not been fully appreciated until now. Our results showed that CD163^{+} MHC II^{+} and TIM4^{-}CD163^{-}MHC II^{+} are derived from bone marrow monocytes. These macrophage subsets were almost virtually lacking in the CCR2$^{-/-}$ animals, as were the macrophage populations previously identified as bone marrow-derived and CCR2-dependent (CD11c^{+} and Ly6C^{+}) (Hill et al. 2018; Li et al. 2010). These data suggest that ATMs are not simply embryonic-derived, as bone marrow contributes to the macrophage resident pool outside the context of disease/injury.

When analyzing the contribution of embryonic-derived macrophages to the tissue resident pool, we found that TIM4^{+}CD163^{+} mainly originates from the yolk sac. Interestingly, this macrophage subset diminishes with age, with a low number of cells at 10-weeks-old, demonstrating a restricted survival of yolk sac-derived macrophages in eWAT. Moreover, our results show that our TIM4^{+} subset had a low amount of YFP^{+} cells, indicating the low contribution of yolk sac-derived cells to this subset. However, as TIM4 is a marker previously used to describe long-lived embryonic-derived cells we were expecting a higher labelling percentage (Chen and Ruedl 2020; Cox et al. 2021). Nevertheless, a similar phenomenon was recognized in the omentum (Etzerodt et al. 2020), where the described TIM4 and CD163 positive population was identified as embryonic-derived, but not the TIM4 positive CD163 negative subset. Possibly, our TIM4^{+}CD163^{+} population is identical to the TIM4 positive subset previously described (Chen and Ruedl 2020; Cox et al. 2021).

We further investigated whether the fetal liver would contribute to the ATM pool using the *Plvap*$^{-/-}$ mouse model. We found that the overall macrophage population was diminished (CD45^{+}CD64^{+}F4/80^{+}), with most of the identified CD206^{+} subsets affected, excluding TIM4^{-}CD163^{-}. The frequency of our TIM4^{+}CD163^{+} subpopulation was almost zero in *Plvap*$^{-/-}$ mice, and TIM4^{+} and CD163^{+} subsets also showed reduced frequencies. These data suggest that fetal liver-derived macrophages may contribute to most steady state ATM subsets, similar to that described in most tissues (Sheng et al. 2015). However, as only two *Plvap*$^{-/-}$ individuals were included in our analysis this last interpretation needs further investigation.

6.2.3 ATMs and metabolic challenge

In diet-induced obesity challenges we confirmed the expected influx of CD11c^{+} macrophages to the eWAT (Weisberg et al. 2003). Remarkably, the CD206^{+} ATM populations did not change their numbers throughout our metabolic challenges,

except the TIM4^{-}CD163^{-} subpopulation that started to merge with CD206^{-} population, suggesting a bone marrow origin for this subset. The fact that the main ATM populations remain steady during the onset of obesity and weight loss proposes a conserved function for these macrophage subsets. Even though it has been established that resident macrophages are the first to proliferate in the context of obesity (Amano et al. 2014; Zheng et al. 2016), it seems that ATMs identified by us do not respond to the tissue's obese environment by proliferating. Instead, we noticed a decrease in the MHC II expression within TIM4^{+}CD163^{+}, TIM4^{+} and, CD163^{+} populations in an obese state. The frequency of MHC II^{-} cells increased in eWAT collected from mice fed with HFD and HFD+Met, resembling the phenotype demonstrated by these macrophage subsets in 2-week-old animals. Interestingly, eWAT starts showing the first fully differentiated adipocytes at 1 week of age.

From 1 week to 2 weeks, the growth of the fat pad is exponential, and the expression of adipogenic and angiogenic genes also increases, evidenced by the high rates of remodeling in this tissue during this stage (Han et al. 2011), which is then stabilized in adult life. Tissue resident macrophages have been identified as key players in regulating the storage of adipocyte lipids (Cox et al. 2021), maintenance of the pre-adipocyte niche (Nawaz et al. 2017; Nawaz and Tobe 2019), clearing the tissue from catabolites (Silva et al. 2019), as well as regulating angiogenesis and adipogenesis during postnatal development (Han et al. 2011). Together these data might explain the MHC II phenotype regression in ATMs, as in an obese state, the eWAT is also under a high rate of tissue remodeling. Macrophages respond to the high tissue remodeling rate by losing MHC II expression. This resembles the macrophages phenotype during the early development of the tissue when the remodeling rate is also high (Han et al. 2011). Notably, this phenotype was rescued if the diet was changed to Chow after the onset of obesity, further supporting the role of ATMs in remodeling of the tissue during fad pad growth. Targeting macrophages that regulate remodeling such as angiogenesis, might be a potential way to treat/prevent obesity (Cao 2010; Nijhawans, Behl, and Bhardwaj 2020).

6.3 The developing iBAT resident macrophages interact with neuronal networks

BAT has a fat burning ability modulated by several cytokines and other stimuli. The cross-talk between brown adipocytes and macrophages has been described as a major mechanism for regulating BAT activity (Campderrós et al. 2019; Cereijo et al. 2018). As an example, cold exposure stimulates brown adipocytes to secrete GDF15 that downregulates the expression of pro-inflammatory genes in macrophages (Campderrós et al. 2019). The perinatal period is when BAT is most active (Liu et al. 2020). However, BAT macrophages have only been described in the mature organ

(Fischer et al. 2017; Gallerand et al. 2021; Pirzgalska et al. 2017; Rosina et al. 2022; Wolf et al. 2017). Here we studied the BAT resident macrophages during the early stages of development.

6.3.1 Macrophages populated embryonic iBAT

At birth, BAT activity is triggered by the exposure to the room temperature (cold) after the intrauterine environment (warm). At this stage, the maintenance of the core body temperature in mice, is mainly achieved by non-shivering thermogenesis from BAT, as the muscles are extremely immature and the onset of shivering gradually starts from around P5 (Agbulut et al. 2003; Arjamaa and Lagerspetz 1979). BAT starts differentiating during embryonic development, around E10, but the iBAT lobes are only distinguishable in the embryo at E15.5 (Shinde et al. 2021). As macrophages have been described to seed embryonic tissues already during primitive hematopoiesis (~E9) (Palis and Yoder 2001), we decided to investigate the presence and the kinetics of macrophages in developing iBAT. The flow cytometric analyses of E15.5 to P56 revealed the presence of macrophages as early as E15.5 and a heterogenic composition of tissue resident macrophages. To our knowledge, this is the first-time that macrophages have been described during the embryonic stages of iBAT. However, a recent study did briefly mentioned the presence of macrophages at E15.5 in iBAT, but the tissue collection also included muscle, indicating a possible inclusion of muscular resident macrophages in their analysis (Rao et al. 2022).

The differential expression of F4/80 during embryonic development allows the preliminary identification of the different origins of macrophages, with F4/80^{Hi} cells originating from the yolk sac, and F4/80^{Int} cells being fetal liver-derived. Additionally, both embryonic F4/80 subsets start merging into a F4/80^{Hi} phenotype after birth, with the infiltration of F4/80^{Int} bone marrow-derived macrophages (Ginhoux and Guilliams 2016; Hashimoto et al. 2013). We found that the F4/80^{Hi} macrophage subset is missing throughout development, which suggests not only a lack of yolk sac-derived macrophages within iBAT but also a rapid turnover of the fetal liver-derived macrophages by bone marrow-derived macrophages during the postnatal period.

6.3.2 Early fetal liver to bone marrow –derived turnover

Indeed, the macrophage monocytic signature, recognized both by flow cytometry and scRNAseq, supports the notion that most macrophages are fetal liver-derived during the pre- and perinatal period. We confirmed this origin by employing fate mapping tracking experiments. Moreover, the macrophage numbers increased exponentially until P7, and then decreased from there onwards. Cell fate mapping

studies confirmed that bone marrow is already contributing to macrophages in iBAT at P7. Bone marrow has been documented as the primary source of macrophages in adult BAT (Gallerand et al. 2021). What is remarkable is the early onset of this turnover – from fetal liver to bone marrow origin, as by 5 weeks of age, we could no longer detect the presence of fetal liver-derived macrophages (data not shown). This macrophage kinetics resembles that described for intestinal mucosa. The embryonic macrophages that populate the lamina propria during the perinatal stage are mainly fetal liver-derived. Around P11, the contribution of bone marrow to the tissue resident pool is already detectable and also coincides with a peak in the macrophage numbers (Bain et al. 2014; Gensollen et al. 2021). This high turnover of macrophages in the gut, with 60% already being of bone marrow-derived cells at around 2 weeks of age (Bain et al. 2014), has been considered to be because of the close contact with external pathogens, thus the need for a constant replenishment from circulating monocytes. However, our results indicate that contact with pathogens is not needed for rapid macrophage turnover in all organs (e.g. BAT), and it will be important to identify the physiological drivers of these kinetic changes in the future.

6.3.3 Fetal liver-derived macrophage deficiency impairs BAT function

The SNS is still developing during the perinatal stages, maturing only around puberty (Scott-Solomon, Boehm, and Kuruvilla 2021; Shinde et al. 2021). We found that fetal liver-derived macrophages interact closely with the developing β-adrenergic nerve fibers already before birth. We hypothesized that fetal macrophages in iBAT might exhibit similar functions to those of the microglia in neurogenesis (Sato 2015). These functions include maintaining the sympathetic innervation of the tissue, and regulating the levels of available catecholamines, as seen in adult iBAT (Pirzgalska et al. 2017; Wolf et al. 2017).

Using the *Plvap*$^{-/-}$ mouse model, which has a normal contribution of the yolk sac and bone marrow –derived macrophages but lacks fetal liver-derived macrophages in most tissues (Rantakari et al. 2016), we evaluated the effect of this deficiency in iBAT function. Our bulk RNA demonstrated downregulation of genes associated with insulin secretion, PPAR signaling, and thermogenesis pathways. The PPAR pathway is essential in BAT, being involved in the generation of new adipocytes, just as PPARγ is essential for the differentiation of adipocytic lineages (Symonds 2013). Additionally, PPARα is integral in the perinatal thermogenic process, promoting fatty acid catabolism and the production of FGF21 in the liver, which induces the activation of the thermogenic transcriptome in BAT (Hondares et al. 2010; Rando et al. 2016). Moreover, at 2 weeks of age, there is an upregulation of PPARα associated with a thermogenic peak. PPARα expression decreases gradually

with age until UCP1 assumes responsibility for the thermogenic processes (Liu et al. 2020). Thus, the downregulation of these three pathways suggests a functional defect in the iBAT of our *Plvap*$^{-/-}$ mouse model. Our infra-red thermogenic analysis of 5-day-old pups supports this evidence, as the *Plvap*$^{-/-}$ mice were significantly cooler than their WT littermates. In general, our *Plvap*$^{-/-}$ analyses proposes a connection with the deficiency of fetal liver-derived macrophages and iBAT functional impairment. This, together with the co-localization of fetal liver -derived macrophages with β-adrenergic fibers, lead us to believe that fetal liver-derived macrophages may have a role in the regulation of perinatal thermogenesis.

6.4 Final remarks

All three fat pads, MG, eWAT, and iBAT, show the high heterogeneity and complex kinetics of tissue resident macrophages. Resident macrophages expressing the CD206 membrane cell marker have been described in all three adipose tissues (Gallerand et al. 2021; Nawaz et al. 2017; Wilson et al. 2022). However, my studies show that the expression of this marker is characteristic to each of the three fat pads as: a specific marker for embryonic-derived macrophages in the mammary gland; a general macrophage marker in steady state WAT; or an ageing acquired macrophage marker in BAT, as during the development of the tissue macrophages do not express CD206. Unfortunately, there are general challenges for comparing the published data to ours, as there is the need to use different sets of surface markers to define specific subsets of macrophages in each fat pad. Moreover, the comparison between fat pads is also difficult due to the high heterogeneity of macrophage phenotypes, with each fat pad having its own specific macrophage subsets.

Furthermore, all three fat pads had a unique combination of macrophages from different origins. Developing MG and iBAT have macrophages primarily originating from fetal liver-derived monocytes. However, fetal liver-derived macrophages in the MG are able to self-maintain untill adulthood, while those in iBAT are already replaced by bone marrow-derived macrophages at 1 week of age. It would be interesting to trace the fetal liver-derived cells in the MG and eWAT with a CCR2 reporter model, to better understand the kinetics and function of these cells throughout the development of these fat pads, especially in WAT as we were not able to identify the origins of all the subsets described.

In my studies, I used the *Plvap*$^{-/-}$ mouse model to study fetal liver-derived macrophage deficiency in the three adipose tissues. The PLVAP molecule is associated with the formation of stomatal and fenestral diaphragms (involved in the vascular permeability) in vascular endothelium (Denzer et al. 2023). *Plvap*$^{-/-}$ mice manifest with several vasculature-related aberrations, leading to a loss of plasma proteins and consequently hyperproteinemia and dyslipidemia. Additionally, during

development *Plvap*$^{-/-}$ mice are smaller than their WT littermates, and present a decrease in fat content (Stan et al. 2012). Accordingly, using *Plvap*$^{-/-}$ mouse model to study adipose tissue development needs to be carefully evaluated. Thus, I employed a specific macrophage depletion strategy when studying MG resident macrophages. It would be interesting to perform similar macrophage depletion experiments in iBAT, as a way to confirm that the *Plvap*$^{-/-}$ mice phenotype in the iBAT's function is associated with a macrophage deficiency and not the endothelial defects of this model. In addition, all three studies would benefit from macrophage depletion with posterior macrophage grafting experiments. This would help to assess whether the ontogeny or the environment is the most important regulator of macrophage phenotype in these tissues. Importantly, the current macrophage depletion protocol affects the organism systemically, which means that all macrophages in the body are depleted. This limits the interpretation of the results, for example, if we depleted macrophages systemically and then studied the effect of this depletion in BAT, the resulting phenotype could be associated to the lack of macrophages in the thermoregulating areas of the brain, causing an altered perception of temperature signaling, which would translate in the absence of thermogenic function in BAT, and not a direct link between lack of macrophages in BAT and thermogenic impairment.

Although each fat pad exhibits specific subsets of macrophages with diverse ontogenies they may have some shared functions. In all three fat pads we have discussed the importance of the tissue resident macrophages for the healthy development of the tissue: in MG the fetal liver-derived macrophages are crucial for the development of the mammary ducts; in WAT our results suggest a key role of tissue resident macrophages in the remodeling of the tissue, either during the healthy or the obesity-associated growth; in BAT, we argue for the possible association of macrophages with the healthy innervation of the tissue, which is essential for the thermogenic function of BAT. Interestingly, both MG and WAT macrophages were able to scavenge particles, thus, it would be interesting to know if in BAT this function is also conserved.

In summary, my results indicate that just as each fat pad has a specific function, their tissue resident macrophages also present different functions.

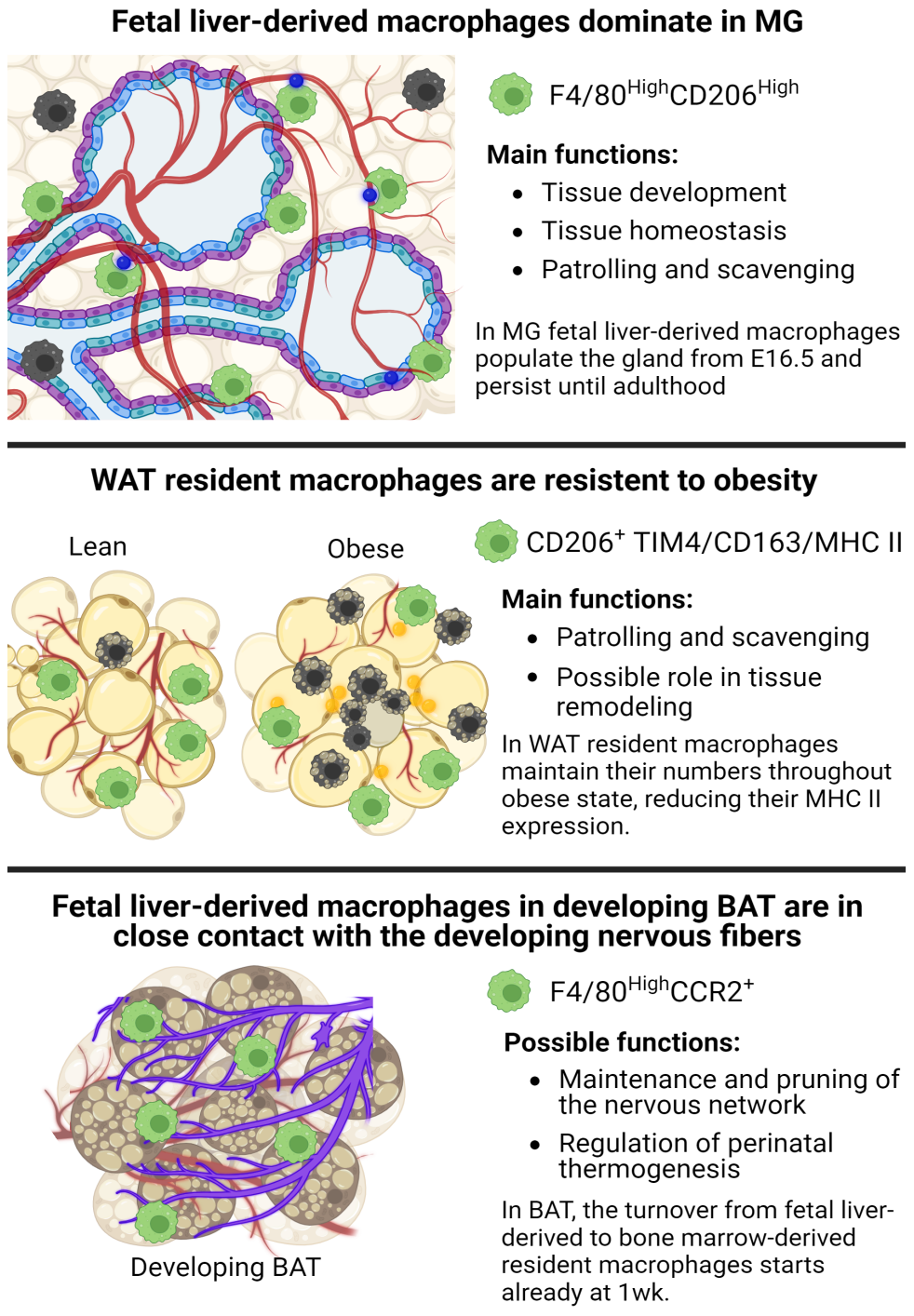

Figure 8 Graphical abstract. The green macrophages represent the tissue resident macrophages studied and the markers that define them. The black macrophages represent other tissue macrophages. Created with BioRender.com

7 Summary/Conclusions

This thesis demonstrates that the macrophages populating the MG, eWAT, and BAT have various origins and are specific to the fat pad. I show that the macrophage heterogeneity and kinetics is highly complex in all three tissues, and that embryonic-derived macrophages seem to be highly involved in the development and function of each tissue.

The main conclusions of the study are the following:

1. MG fetal liver-derived macrophages are already found at E16.5 and are the main macrophage resident population during postnatal life.
2. Fetal-derived macrophages have scavenging functions in the MG and WAT.
3. MG embryonic macrophages are defined by CD206 expression and WAT resident macrophages are defined by CD206, Tim4 and CD163 cell markers, with the $CD206^{+}TIM4^{+}CD163^{+}$ population being fetal-derived.
4. ATMs maintain stable numbers throughout the obese state, responding with a phenotypic shift that resembles the juvenile period.
5. Developing BAT is populated with fetal liver-derived macrophages that closely interact with the neuronal network but are gradually substituted by bone marrow-derived macrophages by the end of the first week of age.

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Inês Alvito Félix

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