On macrophage contributions to tissue homeostasis

New insights on pancreas development and healing of ischemic injury

CARMEN HERRERA HIDALGO
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Abstract

Besides providing host defence against innumerable threats, macrophages display additional key functions for preservation of tissue homeostasis. This thesis includes four studies that explore novel macrophage functions in both the development of islets of Langerhans and healing of ischemic injury in mice.

The aim of Study I was to explore the involvement of pancreatic macrophages in postnatal islet development. We found that neonatal pancreas contained high density of macrophages. Neonatal infections reduced the number of pancreatic macrophages transiently and resulted in both impaired β cell maturation and associated long-standing glucose intolerance. Moreover, clodronate depletion of pancreatic macrophages in the neonate also resulted in long-standing impairment of glucose handling. Together, these results demonstrate that macrophages in the neonatal pancreas are important for maturation of islet function.

We then wanted to understand how macrophages contribute to healing of ischemic injury based on the observation that they accumulate at perivascular positions following ischemia. We found that blood flow at the site of ischemia was regulated by perivascular macrophages in an iNOS-dependent manner, which could be targeted to increase tissue healing (Study II). Next, we investigated if these perivascular macrophages trans-differentiate into mural cells. By lineage tracing, we found that macrophages undergo a phenotype shift at the site of ischemic injury, as they down-regulated the expression of myeloid cell lineage markers (CD45/CX3CR1/CD11b) and upregulated the expression of the mural cell marker PDGFRβ (Study III). Lastly, we addressed if macrophages are involved in vascular remodelling important for tissue normalization by pruning excessive vessels at the site of injury. Indeed, MMR⁺-macrophages were found to support vessel pruning during vascular normalization at late phases of healing (Study IV).

In conclusion, this thesis reveals novel functions of macrophages as they support postnatal maturation of the insulin-producing β cells of the pancreas, as well as restore blood flow and normalize the vasculature during healing of ischemic injuries. Together, the studies in this thesis contribute to illustrating the ample and diverse macrophage curriculum and how macrophage skills cooperate to ensure homeostasis.

Keywords: macrophages, islets of Langerhans, β cell function, postnatal β cell maturation, hindlimb ischemia, blood flow regulation, mural cells, vessel pruning, diabetes mellitus, ischemic diseases

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urn:nbn:se:uu:diva-395535 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-395535)
To my parents
and to my grandpa Joaquin
List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


IV  **C. Herrera–Hidalgo**, K, Parv, B. Laviña, M. Phillipson. Macrophages contribute to vessel normalization during healing of ischemic injury. *Manuscript*

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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>AGM</td>
<td>Aorta–gonads–mesonephros</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>CD11b</td>
<td>CD11 Antigen–Like Family Member B</td>
</tr>
<tr>
<td>CD169</td>
<td>CD11 Antigen–Like Family Member B</td>
</tr>
<tr>
<td>CD31</td>
<td>CD31 Antigen; Platelet And Endothelial Cell Adhesion Molecule</td>
</tr>
<tr>
<td>CD45</td>
<td>C–X–C Motif Chemokine Receptor 4</td>
</tr>
<tr>
<td>CD49d</td>
<td>CD49 Antigen–Like Family Member D</td>
</tr>
<tr>
<td>CD68</td>
<td>CD68 Antigen; Scavenger Receptor Class D, Member</td>
</tr>
<tr>
<td>Cdc42</td>
<td>Cell division control protein 42 homolog</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony formation units</td>
</tr>
<tr>
<td>Cldn5</td>
<td>Claudin 5</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CSF1</td>
<td>Colony–stimulating factor 1</td>
</tr>
<tr>
<td>CSF1R</td>
<td>Colony–stimulating factor 1 receptor</td>
</tr>
<tr>
<td>CX3CR1</td>
<td>C–X3–C Motif Chemokine Receptor 1</td>
</tr>
<tr>
<td>CXCL12</td>
<td>C–X–C Motif Chemokine Ligand 12</td>
</tr>
<tr>
<td>CXCR4</td>
<td>C–X–C Motif Chemokine Receptor 4</td>
</tr>
<tr>
<td>DAMPs</td>
<td>Damage Associates Molecular Pattern</td>
</tr>
<tr>
<td>E</td>
<td>Embryonic day</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>FOXA2</td>
<td>Forkhead Box A2</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GLUT2</td>
<td>Glucose Transporter Type 2</td>
</tr>
<tr>
<td>GSIS</td>
<td>Glucose–stimulated insulin secretion</td>
</tr>
<tr>
<td>GTT</td>
<td>Glucose tolerance test</td>
</tr>
<tr>
<td>HIF–1</td>
<td>Hypoxia–inducible factor 1</td>
</tr>
<tr>
<td>HSC</td>
<td>Hematopoietic Stem Cell</td>
</tr>
<tr>
<td>i.p.</td>
<td>Intra–peritoneal</td>
</tr>
<tr>
<td>i.v.</td>
<td>Intra–venous</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin–like factor</td>
</tr>
<tr>
<td>IL–</td>
<td>Interleukin</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible</td>
</tr>
<tr>
<td>INS</td>
<td>Insulin gene</td>
</tr>
<tr>
<td>Ki67</td>
<td>Marker of proliferation Ki–67</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>LXR</td>
<td>Liver X nuclear receptor</td>
</tr>
<tr>
<td>MAF</td>
<td>MAF BZIP transcription factor</td>
</tr>
<tr>
<td>MAO</td>
<td>Metabolically abnormal obese</td>
</tr>
<tr>
<td>MARCO</td>
<td>Macrophage receptor MARCO</td>
</tr>
<tr>
<td>MCP–1</td>
<td>Monocyte chemoattractant protein–1</td>
</tr>
<tr>
<td>MFI</td>
<td>Mean fluorescent intensity</td>
</tr>
<tr>
<td>MHCII</td>
<td>major histocompatibility complex class II</td>
</tr>
<tr>
<td>MMP–9</td>
<td>Matrix metalloproteinase 9</td>
</tr>
<tr>
<td>MMR</td>
<td>Macrophage mannose receptor</td>
</tr>
<tr>
<td>MNO</td>
<td>Metabolically normal obese</td>
</tr>
<tr>
<td>NG2</td>
<td>Chondroitin sulfate proteoglycan NG2</td>
</tr>
<tr>
<td>Ng3</td>
<td>Neurogenin 3</td>
</tr>
<tr>
<td>Nkx2–2</td>
<td>NK2 homeobox 2</td>
</tr>
<tr>
<td>Nkx6–1</td>
<td>NK6 homeobox 1</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>ns</td>
<td>Not significant</td>
</tr>
<tr>
<td>PAD</td>
<td>Peripheral artery disease</td>
</tr>
<tr>
<td>PAX6</td>
<td>Paired Box 6</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet–derived growth factor</td>
</tr>
<tr>
<td>PDGFRβ</td>
<td>Platelet–derived growth factor receptor β</td>
</tr>
<tr>
<td>PDX1</td>
<td>Pancreatic and duodenal homeobox 1</td>
</tr>
<tr>
<td>PPAR</td>
<td>Peroxisome proliferator activated receptor</td>
</tr>
<tr>
<td>PPRs</td>
<td>Pattern recognition receptors</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>S. aureus</td>
<td>Staphylococcus aureus</td>
</tr>
<tr>
<td>SCF</td>
<td>Stem cell factor</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard deviation of the mean</td>
</tr>
<tr>
<td>T1D</td>
<td>Type 1 diabetes</td>
</tr>
<tr>
<td>T2D</td>
<td>Type 2 diabetes</td>
</tr>
<tr>
<td>TAMs</td>
<td>Tumor associated macrophages</td>
</tr>
<tr>
<td>TGFB1</td>
<td>Transforming growth factor β 1</td>
</tr>
<tr>
<td>Tie2</td>
<td>Tyrosine–Protein Kinase Receptor Tie2</td>
</tr>
<tr>
<td>TNFa</td>
<td>Tumor Necrosis Factor α</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VEGFR</td>
<td>Vascular Endothelial Growth Factor Receptor</td>
</tr>
<tr>
<td>WNT7B</td>
<td>Wnt Family Member 7B</td>
</tr>
</tbody>
</table>
Introduction

Whenever the organism enjoys immunity, the introduction of infectious microbes is followed by the accumulation of mobile cells, of white corpuscles of the blood in particular, which absorb the microbes and destroy them.

Ilya Mechnikov (1845–1916)

For survival, all biological systems relay on the maintenance of key physiological parameters within a desired range. Compromised equilibrium of such parameters is incompatible with health, thus resulting in disease onset. The concept of homeostasis was proposed in the 19th century by Claude Bernard (1813–1878) to describe the array of uncountable physiological mechanisms of the living organism, that work together to maintain a stable internal milieu regardless of external (and internal) disturbance. The term homeostasis was later minted by Walter Cannon in the 20th century.

For long, our knowledge of the contribution of the immune system to homeostasis was primarily limited to its ability to keep infections at bay. However, it is today widely accepted that the immune system, comprising anatomical barriers, cells and molecular effectors spread throughout the body, plays additional vital roles in maintaining tissue integrity. For instance, the immune system contributes to organogenesis, wound healing and prevention of tumor formation. To fulfill all these tasks and match the different scenarios it faces, the immune system is armed with a great sensory capacity and the ability to compose an immune response tailored to the specific insult.

The work presented in this thesis delineates novel functions of a specific cell of the immune system, the macrophage, in preserving tissue homeostasis by: 1) supporting the postnatal maturation of the insulin–producing β cells of the pancreas, and 2) promoting blood flow restoration and vascular normalization during healing of ischemic injuries.
Macrophages

Macrophages were first described in the late 19th century by Ilya Mechnikov as an evolutionary conserved cell type specialized in engulfing and eliminating foreign intruders. He minted the name based on his observation of a group of big cells (makros) that migrated towards and encapsulated (phagos) exogenous particles in the starfish larvae. The discovery of these professional phagocytes formed the basis of the cellular theory of immunity. This theory raised much controversy at the time, when immune protection was believed to involve only body fluids and soluble substances (the humoral theory). This work earned Mechnikov, together with Paul Ehrlich, the Nobel Prize in Physiology or Medicine in 1908 "in recognition of their work on immunity", just as Mechnikov’s theory gained acceptance within the scientific community as a synergic immune mechanism to humoral immunity. Already then, Mechnikov proposed that macrophages were important for both development and tissue homeostasis, in addition to their role as sentinels, due to his observation that these phagocytes scavenged obsolete cells throughout the metamorphosis process from tadpole to adult frogs by the same engulfing mechanism (Tauber, 2003).

It is now well-established that macrophages from different origins are ubiquitously spread throughout the tissues of the body in vertebrates. Tissue-resident macrophages are long-lived and highly diverse. Distinct macrophage populations display specialized functions, such as matrix resorption in the bone (Yoshida et al., 1990) or clearance of senescent red blood cells and subsequent iron recycling by red pulp macrophages in the spleen (Kohyama et al., 2009; Mebius and Kraal, 2005). The vast macrophage heterogeneity was elegantly depicted by the Immunological Genome Project (Gautier et al., 2012) who uncovered minimal transcriptional overlap between different tissue-resident macrophages. This heterogeneity is a reflection of macrophage adaptation to the niche where they reside and of their ability to switch phenotypes in response to microenvironmental cues. Further, if and how macrophage origin dictate their identity and function is a matter of discussion in the field (Ginhoux and Guilliams, 2016; Hashimoto et al., 2013; Hoeffel and Ginhoux, 2018; Wynn et al., 2013; Yona et al., 2013).

Macrophage ontogeny

In mice, the first primitive macrophage arises from an extra-embryonic progenitor from the blood islands of the yolk sac at embryonic day (E) 10.5 (Figure 1) (Hoeffel and Ginhoux, 2018). A subsequent wave of hematopoiesis occurs from definitive hematopoietic stem cells (HSCs) emerging at the aorta-gonads-mesonephros (AGM) and the fetal liver, that together take over generating macrophages, in this case from a monocyte intermediate (Figure 1) (Hoeffel and Ginhoux, 2018). During organogenesis, macrophages seed the
microglia in the brain (Alliot et al., 1999; Ginhoux et al., 2010; McGrath et al., 2003; Yona et al., 2013), the Langerhans cells of the skin (Ginhoux and Merad, 2010; Hoeffel et al., 2012) and the lung alveolar macrophages (Guilliams et al., 2013), among other tissue–resident macrophages. Interestingly, most tissue–resident populations originate from an embryonic precursor, are self–maintained and turn over locally via proliferation under steady–state conditions (Epelman et al., 2014; Ginhoux and Guilliams, 2016; Gomez Perdiguero et al., 2015; Hashimoto et al., 2013; Hoeffel and Ginhoux, 2018; Samokhvalov et al., 2007; Wynn et al., 2013; Yona et al., 2013).

Figure 1. Location of macrophage progenitors during development. Macrophages from different origins seed the tissues of the body throughout development. The first macrophage emerges from a hematopoietic precursor from the blood islands in the yolk sac. These primitive macrophages infiltrate the developing central nervous system and give rise to microglia in the brain (McGrath et al., 2003). Subsequent waves of hematopoiesis generate macrophages from a monocyte intermediate at the proper hemogenic endothelium of the AGM and the fetal liver in the embryo, or at the bone marrow after birth (Hoeffel and Ginhoux, 2018).

Following birth, bone marrow assumes the main responsibility for definitive hematopoiesis (Figure 1). Thereby, bone marrow/monocyte–derived macrophages replenish some resident macrophage populations, including those of tissues with high cellular turnover such as the gut (Bain et al., 2014) and the dermis (Tamoutounour et al., 2013). The circulating monocyte pool also provides the organism with macrophage precursors as they infiltrate tissues when summoned once intruders or injury threatens tissue homeostasis and survival, as part of the body’s first line of defense.

Colon–stimulating factor 1 receptor (CSF1R) is essential for macrophage differentiation, proliferation and survival and thereby, for the development of most tissue–resident macrophages. Indeed, mice harboring a null mutation of the Csf1 gene (Csf1−/− mice) lack most tissue–resident macrophages and present profound developmental abnormalities: elevated perinatal mortality, osteopetrosis, growth defects, distorted glucose homeostasis and reduced mass of insulin–producing β cells, among others (Banaei-Bouchareb et al., 2004;
Dai et al., 2002; McKercher et al., 1996; Naito et al., 1991). Altogether, these evidences illustrate the vast heterogeneity of macrophage functions that contribute to countless physiological and developmental processes.

Macrophage functions
As mentioned above, macrophages are integral components of tissues. They are central to preserving the host’s integrity by clearing pathogens and dying or damaged cells during development and adult life. Educated by cues from the surroundings, macrophages assume specialized phenotypes and contribute to the appropriate functioning of their tissue of residence. These phenotypic adaptations were first understood as dichotomous, and macrophages were classified based on their activation state as M1, the classical microbe assassin, and M2, the alternatively activated macrophage specialized in tissue remodeling (Gordon, 2003; Sica and Mantovani, 2012). Although this classification was important to understand macrophage plasticity, it is today accepted that it does not fully reflect the functional complexity of tissue–resident macrophages. The present section of this thesis modestly discusses some aspects of macrophage plasticity and of their vast functional repertoire.

Immune sentinels
Macrophages are motile cells endowed with the ability to detect and phagocytose pathogens and to orchestrate an inflammatory response to intruders by secreting soluble immune mediators and antimicrobial effectors. For pathogen detection, macrophages are well–equipped with a plethora of complement specific, Fc and pattern recognition receptors (PPRs). The proficient phagocytic ability of macrophages relays on a specialized lysosomal compartment armed with key proteases and bactericidal activity, the phagosome, where pathogens are inactivated once engulfed (Stuart and Ezekowitz, 2005). In addition, upon pathogen recognition, macrophages secrete an array of antimicrobial molecules that are essential for host defense, such as nitric oxide (NO) and reactive oxygen species (ROS). Macrophages subsequently mount a complex immune response: secretion of Interleukin 1β (IL–1β) and Tumor Necrosis Factor α (TNFα) induce the expression of adhesion molecules at the apical side of the endothelium as well as vessel dilation, together resulting in immune cell recruitment to the site of infection. Combined, these functions are essential in the protection against infections (reviewed in Franken et al., 2016).

Tissue repair
Tissue damage results in cell death that initially activates innate immune responses somewhat similar to those triggered by pathogens. Macrophages dis-
play great sensory capacity to identify injury through Damage Associates Molecular Pattern receptors (DAMPs) and are promptly recruited at the early stage of tissue repair. One of the better studied examples of macrophage contributions to healing is the case of skin wounds, where macrophages are indispensable for the various phases of the healing process, in particular for re-epithelialization, tissue revascularization and scar formation (Lucas et al., 2010; Öhnstedt et al., 2019; Vannella and Wynn, 2017). Initially, macrophages release pro-inflammatory molecules at the site of the wound, including IL–1, IL–6, TNF–α, NO, and ROS for antimicrobial defense (Murray and Wynn, 2011). Macrophages subsequently upregulate the expression of growth factors such as platelet derived growth factor (PDGF), insulin–like factor 1 (IGF–1) and vascular endothelial growth factor (VEGF) to promote cell proliferation and blood vessel formation and, in this way, relieve the prevailing state of local hypoxia (Berse et al., 1992; Chujo et al., 2009; Liu et al., 2016; Patel et al., 2013; Rappolee et al., 1988; Shimokado et al., 1985). In addition, macrophages promote wound contraction and closure by inducing myofibroblast differentiation in a transforming growth factor β 1 (TGFβ1)–dependent manner and promote extracellular matrix remodeling (Akhurst and Hata, 2012). As healing progresses, macrophages shift towards pro-resolution phenotypes characterized by the expression of IL–10 and TGF–β1. This way, they mitigate the inflammation and prevent collateral damage of the surrounding tissue (Khalil et al., 1989; Shouval et al., 2014; Vannella and Wynn, 2017).

**Tissue vascularization**

Tissue vascularization occurs during both organogenesis and healing to warrant oxygen and nutrients supply. Macrophages are essential to the development of the vasculature and have been shown to support sprouting and anastomosis (endothelial cell fusion) by promoting endothelial cell contacts and bridging their fusion in a VEGF–dependent manner in the central nervous system (CNS) (Fantin et al., 2010; Rymo et al., 2011). In addition, during healing of ischemic injuries, macrophages promote arteriogenesis in a monocyte chemoattractant protein–1 (MCP–1)–dependent manner (Heil et al., 2006; Schaper et al., 1976) and angiogenesis (Corliss et al., 2016; Sunderkötter et al., 1994). To induce angiogenesis, they release factors that induce proliferation and migration of endothelial cells, such as VEGF–A, PDGF and IGF–1 in addition to matrix remodeling proteins (Berse et al., 1992; Liu et al., 2016; Patel et al., 2013; Shimokado et al., 1985). Indeed, tumor associated macrophages (TAMs) are recruited to hypoxic sites where they induce the angiogenic switch allowing early tumors to progress.

In addition to promoting vessel growth, macrophages drive vessel regression in different settings. Macrophages has been shown to clear apoptotic endothelial cells as the hyaloid vessels regress (Mitchell et al., 1998). By production of WNT7B, Tie2–expressing macrophages target endothelial cells to apoptosis during hyaloid vessel development (Rao et al., 2007). In the adult
mouse, macrophages prune the vessel surplus that forms to compensate for injury–induced hypoxia during healing of skin wounds (Gurevich et al., 2018). In addition, macrophages are involved in other physiological vascular regression processes in adults, such as luteolysis during the menstruation cycle (Thiruchelvam et al., 2013).

**Functional specification of macrophage subsets**

As previously mentioned, next to these prevalent features, tissue–resident macrophage populations have unique identities and functions that are shaped by environmental cues from their niche of residence. In an effort to illustrate macrophage heterogeneity, some of their specialized tissue–specific functions are described below and represented in Figure 2.

**Splenic macrophages**

In the spleen, four distinct macrophage subsets are found at distinct micro–anatomical locations. Macrophages in the red pulp clear damaged or aged erythrocytes at the “open” circulation system found at the venous sinusoids, and efficiently recycle the resultant iron from the heme group in hemoglobin (Kohyama et al., 2009; Mebius and Kraal, 2005). In the white pulp, macrophages preserve local homeostasis by phagocytosis of dead, activated, lymphocytes (Davies et al., 2013), which is believed to be important in suppressing self–reactivity to apoptotic cells (McGrath et al., 2003). Two additional macrophage subtypes locate at the boundaries of the red and white pulps: the metallophilic, CD169–expressing macrophages and around them, the MARCO–expressing marginal zone macrophages. These populations are specialized in immune surveillance of the circulating blood and are essential in the pivotal task of eliminating blood–borne pathogens (Geijtenbeek et al., 2002; Kang et al., 2004; Karlsson et al., 2003).

**Brain microglia**

Microglia are yolk sac derived macrophages in the brain. They display highly dynamic sensory protrusions that probe the surrounding brain parenchyma (Davalos et al., 2005; Nimmerjahn et al., 2005) and physically interact with synapses. However transient, these contacts have been shown to modulate neuronal activity (Tremblay et al., 2010). Indeed, mice deficient in C–X3–C Motif Chemokine Receptor 1 (CX3CR1), a receptor prominently expressed by microglia and other tissue macrophages, present impaired synaptic transmission and behavioral phenotypes associated to neurodevelopmental disorders. During embryogenesis, these resident macrophages both provide crucial trophic support for appropriate neuronal partnering and regulate cell apoptosis and survival in the developing CNS (Frost and Schafer, 2016; Schafer and Stevens, 2015). During postnatal growth of the brain, microglia actively shapes the neuronal circuit by pruning futile synapses and phagocytosing apoptotic neurons. This is characterized in mouse models where microglia is
deficient of CX3CR1 or of functional complement receptors, which lack efficient synaptic pruning and present defects in neuronal connections (Paolicelli et al., 2011; Schafer et al., 2012; Takahashi et al., 2005).

**Osteoclasts**

Osteoclasts are bone–resident multinucleated macrophages specialized in bone resorption. Thereby, they assume a main responsibility in maintenance, repair and remodeling of bone tissue. When the equilibrium between bone formation and degradation is unbalanced, osteoporosis (loss of bone mass) or osteopetrosis (excess bone mineralization) develop. Indeed, CSF1op/op mice have an abnormally dense bone structure, osteopetrosis, and concomitant impediment of bone growth (Yoshida et al., 1990).

![Figure 2. Representation of the functional diversity of tissue–resident macrophages. A) Four types of functionally distinct macrophages inhabit the spleen. B) Osteoclasts are macrophages of the bone and are specialized in matrix resorption, which is important for bone remodeling and growth. C) Microglia are brain macrophages with important functions for neuronal circuit patterning.](image)

**Other specialized functions of tissue–resident macrophages**

Additional specialized functions of tissue–resident macrophages subsets have been described. For instance, alveolar macrophages maintain lung homeostasis by clearance of surfactant (Suzuki et al., 2008). Recently, a group of macrophages, which that located around the atrioventricular node (AV node), have been shown to modulate its depolarization and thereby, the electrical activity of the heart (Hulsmans et al., 2017).

Macrophages in endocrine tissues have been reported to be associated with the blood vasculature and to project elongated protrusions in an apparent effort to connect different cell types (Unanue, 2016). Emil R. Unanue and colleagues recently defined three distinct populations of resident macrophages in the adult mouse pancreas, which share the common expression of CD68 and F4/80. Macrophages in the islets of Langerhans originate from definitive hematopoiesis, show a transcriptional signature typical of an M1 activation phenotype during steady state in adult mice (Calderon et al., 2015) and are capable of sensing cues from plasma, including microbial products (Ferris et al., 2017; Zinselmeyer et al., 2018). Interestingly, they are believed to be activated at
weaning in the non-obese diabetic mouse model (Ferris et al., 2017). In contrast, the two different subtypes of interacinar stromal macrophages correlate with an M2like activation profile (Calderon et al., 2015). The CSF1op/op mice, which have severe scarcity of most tissue macrophages, display reduced insulin mass and disturbed glucose homeostasis, together with osteopetrosis, growth retardation and several additional developmental defects (Banaei-Bouchareb et al., 2004; Naito et al., 1991). How these distinct macrophage subtypes contribute to islet development as well as their implications in different pancreatic diseases such as diabetes or pancreatitis remains unknown.

Macrophages in disease

Under certain circumstances and underlying diseases macrophages instead contribute to disease progression. Indeed, defective resolution of the inflammatory response to injury results in fibrosis, sustained by hyper–activated macrophages that become pro–fibrotic.

In adipose tissues, macrophages provide trophic support to adipocytes and maintain metabolic homeostasis during steady–state conditions. They represent around 10% of all stromal cells in this tissue and increase up to 60% during metabolic syndrome and obesity. Driven by hypoxia created by adipose tissue expansion during weight gain, macrophages of the visceral adipose tissue shift phenotype from IL–10 producing to expressing iNOS, IL–6 and TNFα. This phenotype switch generates a systemic inflammatory profile that contributes to the obesity–induced peripheral insulin resistance observed in diabetes (Chawla et al., 2011; Fujisaka et al., 2013; Xu et al., 2003). In addition, macrophages also accumulate in high numbers in the islets of Langerhans of individuals with diabetes. By secreting IL–1β, these macrophages contribute to islet inflammation and cause β cell stress and dysfunction (Eguchi and Nagai, 2017).

Even if macrophages are initially anti–tumorigenic, when exposed to malignant environments TAMs often support tumor progression and metastasis (Biswas and Mantovani, 2010; Qian and Pollard, 2010). By secreting different factors that include CXCL12, IL–10, IL–4 and IL–13, cancer cells recruit and educate TAMs to transition towards an immunosuppressive phenotype, exploited by the tumor to, for instance, escape immune recognition. Tumors also benefit from the excellent tissue remodeling abilities of macrophages, which by releasing matrix metalloproteases loosen up the extracellular matrix creating an ideal environment for cell invasion and metastasis. In addition, Tie–2 expressing TAMs support the angiogenic switch by production of VEGF and other angiogenic mediators, promoting tumor hyper–vascularization (Condeelis and Pollard, 2006; DeNardo et al., 2010; Hanahan and Coussens, 2012; Wynn et al., 2013).
Diabetes mellitus

Diabetes mellitus is a group of chronic metabolic diseases characterized by hyperglycemia, the persistence of abnormally elevated glucose levels. Diabetes develops when the insulin–producing β cells of the islets of Langerhans in the pancreas fail to meet the body’s insulin demands. This can be the consequence of progressive autoimmune destruction of β cells, as in type 1 diabetes (T1D) (Yoon and Jun, 2005), or of β cell insufficiency coupled with peripheral insulin resistance, as in type 2 diabetes (T2D) (Rahier et al., 2008; Stumvoll et al., 2005). Diabetes is a global pandemic, the estimated prevalence has increased four times over the last 35 years from 108 million in 1980 to 422 million in 2014, with a present predicted annual societal cost of 850 billion USD (Ogurtsova et al., 2017).

T2D is the most common form of the disease and accounts for more than 90% of all diabetes cases. It is a multifactorial disease involving environmental, genetic and epigenetic risk components and is strongly associated with obesity, unhealthy diet, sedentary lifestyles and aging. Insulin resistance, the inability of tissues to effectively respond to this hormone, is the main hallmark of T2D but is also detected in pre–diabetic individuals, a high risk metabolic condition that precedes T2D disease onset (Abdul-Ghani et al., 2006; DeFronzo et al., 2015; Stumvoll et al., 2005). However, overt T2D develops as a result of β cell failure and the subsequent inability to compensate the increase insulin demands resulting from the state of peripheral resistance (DeFronzo et al., 2015; Stumvoll et al., 2005). The ability to adapt to this increased insulin demand determines whether an individual develops diabetes and, in early stages of the disease, a compensatory increase in β cell mass and in insulin secretion are observed (Saisho et al., 2013). Failure to adapt results from β cell exhaustion and associated stress that progresses into β cell degeneration and loss of insulin positive mass in the pancreas (Alejandro et al., 2015). Reduced insulin mass in persons with T2D occurs due to β cell apoptosis and/or silencing of the insulin promoter (Butler et al., 2003), with concomitant loss of adequate glycemic control. Impaired β cell function also accounts for insulin insufficiency. Indeed, decreased glucose sensitivity of β cells and concomitant decline of glucose–stimulated insulin secretion (GSIS) are observed in persons with T2D and is an accurate predictor of the disease progression (Ferrannini et al., 2011).

Even if obesity is the highest risk factor for T2D, a proportion of obese persons are classified as metabolically normal (MNO) and have a significantly lower risk than metabolically abnormal persons with obesity (MAO) to develop diabetes (Appleton et al., 2013; Meigs et al., 2006). The reasons for inter–individual differences in susceptibility to T2D are not fully clarified. Thus, improved understanding of the disease etiology and risk factors are expected to allow for conception of novel therapeutic strategies for diabetes prevention and/or treatment. Along these lines, in this thesis I explored whether
immune challenges occurring during β cell development, such as bacterial infections, interfere with the acquisition of a functionally mature, adult β cell pool (see Study I).
The pancreas

The pancreas is an organ that functions as both an endocrine and exocrine gland. The exocrine pancreas constitutes nearly 95% of the pancreatic mass and is composed of clusters of acinar cells (acini) connected through a system of ducts that drains the acini–secreted digestive enzymes, bicarbonate and mucins into the duodenum (Khonsary, 2017). The endocrine portion consists of endocrine cells that bundle into islets of Langerhans, which are interspersed throughout the acini. The islets account for only 2% of the total pancreas mass and are essential for the regulation of glucose metabolism and the maintenance of appropriate glucose levels in blood through the secretion of specialized hormones (Khonsary, 2017).

During mouse embryogenesis, pancreatic precursors characterized by the expression of pancreatic and duodenal homeobox 1 (PDX1) bud from the foregut endoderm (E 9.5) and this invagination evolves into a tubular structure, a process that is dependent on cell division control protein 42 homolog (Cdc42) (Benitez et al., 2012; Kesavan et al., 2009). Driven by tubulogenesis and branching morphogenesis, the pancreas grows and develops into lobules and endocrine cells differentiate as they assemble into highly structured, vascularized and innervated mini–organs: the pancreatic islets (Figure 3) (Benitez et al., 2012; Hick et al., 2009; Villasenor et al., 2010).

![Figure 3. Pancreas organogenesis. The murine pancreas starts budding from the foregut endoderm from a subset of cells that express Pdx1 (E 9.5). The subsequent formation of micro–lumens (E11) initiates the formation of the ductal tree (E13). From E13, the developing pancreas grows into numerous lobules through branching morphogenesis. Acinar cells differentiate and expand and endocrine cells specify and cluster into islets of Langerhans scattered throughout the exocrine portion of the pancreas.](image-url)
Endocrine pancreas development

During embryonic development, ductal Neurogenin (Ngn3)–expressing endocrine progenitors differentiate into α, β, δ, ε or PP cells committed to produce the respective hormones: glucagon, insulin, somatostatin, ghrelin or pancreatic polypeptide (Benitez et al., 2012; Bonner-Weir et al., 2016; Desgraz and Herrera, 2009; Gu et al., 2002; Wang et al., 2010). Endocrine cells cluster together and form the islets of Langerhans that are scattered throughout the pancreas. The islets develop an intricate system of endocrine inter–cellular connections and become innervated and highly perfused by a dense intra–islet vasculature to together enable fine–tuned regulation of blood glucose (Henderson and Moss, 1985; Jansson, 1994; Jansson and Carlsson, 2002; Moldovan and Brunicardi, 2001; Rodriguez-Diaz et al., 2011; Roscioni et al., 2016).

Islet development spans through the postnatal period with a prominent expansion of β cell numbers. Neonatal β cells are immature and postnatal development is necessary for their functional maturation, as they improve their capacity for GSIS and increase their insulin reservoirs (Figure 4) (Benitez et al., 2012; Bonner-Weir et al., 2016). Thus, driven by the detachment from maternal insulin at birth and next by the exposure to new energy sources and changing requirements, the postnatal period is decisive for the metabolic adaptation of the newborn to attain adult capacity to manage glucose. Thereby, disturbances of these postnatal processes are likely to give rise to a maladaptive β cell mass and thus, to account for inter–individual susceptibility to diabetes. Interestingly, this has indeed been described in cases of experimental (Swenne et al., 1987, 1992) or clinical (Bhatia et al., 1995) early–life malnutrition. During adulthood, β cell mass is delicately regulated to satisfy the host’s changing metabolic demands within certain limits: it expands upon increased calorie intake and obesity, as well as during pregnancy (Butler et al., 2003; Saisho et al., 2013).

The MAFA/B basic leucine zipper family of transcription factors is essential for postnatal β cell functional maturation and their expression by endocrine cells is regulated in a fine–tuned spatiotemporal fashion in the developing islet. In β cells, MAFA expression, which is controlled by the β cell–specific transcription factors PDX1, Forkhead Box A2 (FOXA2), Paired Box 6 (PAX6), NK6 Homeobox 1 (NKX6–1) and NK2 Homeobox 2 (NKX2–2), substitutes MAFB at birth (Artner et al., 2010; Hang and Stein, 2011; Nishimura et al., 2006). In turn, MAFA induces the expression of β cell specific genes essential for glucose sensing and stimulated release, including Insulin (INS), PDX1 and Glucose Transporter Type 2 (GLUT2) (Artner et al., 2010; Hang et al., 2014; Wang et al., 2007). Indeed, MAFA has been shown to regulate the secretion of insulin in response to glucose (Zhang, 2005; Artner, 2010), its overexpression upregulates insulin secretion (Aguayo-Mazzucato et al., 2015) and knock–out mice for MAFA harbor immature β cells and develop
diabetes (Zhang et al., 2005). Remarkably, mutations in the MAFA gene have been found in persons with diabetes mellitus (Iacovazzo et al., 2018).

Figure 4. Postnatal maturation of insulin producing β cells. Neonatal β cells exhibit high proliferation, which declines with postnatal β cell development. High proliferation seems associated with β cell immature phenotypes and ectopic overexpression of mitogenic c–Myb in adult β cells renders them immature (Puri et al., 2018). The neonatal GSIS capacity is weak, β cells respond poorly to increasing glucose levels and present delayed cessation of insulin production in response to a decrease in the glucose levels (Benitez et al., 2012; Bonner-Weir et al., 2016).

It is anticipated that a better understanding of the mechanisms controlling β cell development and β cell mass regulation will open new therapeutic avenues allowing for induction of strategic preservation and/or regeneration of insulin–producing β cell mass. I have identified macrophages as interesting targets, since they participate in fetal development of numerous organs including the pancreas where they have been reported to accumulate during the time of islet maturation (Charré et al., 2002). In addition, macrophages also accumulate in adult pancreas in response to islet injury, where they promote β cell proliferation (Brissova et al., 2014). Further, co–culturing of macrophages with embryonic pancreatic tissues depleted of mesenchymal cells was demonstrated to positively affect islet maturation following transplantation under the kidney capsule (Mussar et al., 2014). In Study I in this thesis, I investigated how distortion of the pancreatic resident macrophage population during the neonatal period affects β cell maturation and long–term function.
Ischemic diseases

Ischemic diseases enclose cardiovascular conditions including angina, myocardial infarction, peripheral artery disease (PAD) and cerebrovascular diseases such as stroke, which are characterized by insufficient blood perfusion and concomitant inadequate tissue oxygenation. Accordingly, tissue ischemia and local injury develop as the blood supply fails to meet the metabolic demands. Tissue ischemia also develops as a consequence of trauma or during organ transplantation. Worldwide, PAD is the most common form of ischemic diseases and a leading cause of cardiovascular–related mortality and morbidity (Criqui Michael H. and Aboyans Victor, 2015). The most prominent underlying cause of PAD is atherosclerosis: progressive narrowing of arteries due to abnormal lipid deposition in the inner arterial wall that occurs in persons with dyslipidemia. Indeed, ischemic cardiovascular diseases are highly associated with obesity and diabetes, both characterized by systemic metabolic imbalance that is deleterious to the vasculature.

Oxygen is central to life and evolution has granted us with the ability to adapt to changes in oxygen availability to, for instance, cope with physical activity or life at different altitudes (Horscroft et al., 2017). The groundbreaking discovery of the fundamental molecular mechanisms that allow the cells of the body to sense and adapt to low oxygen bioavailability earned William G Kaelin, Sir Peter Ratcliffe and Gregg L Semenza the Nobel Prize in Physiology or Medicine this year (2019), whose award announcement I was listening to with excitement while writing these very lines. During chronic ischemia, the fundamental mechanisms of acclimation fail to restore adequate tissue perfusion, resulting in persistent damage that can result in loss of tissue function. Ischemic diseases present a rampant prevalence worldwide and despite the efforts to design strategies aiming at restoring tissue perfusion to rescue ischemic diseases’ ominous effects, current therapeutic approaches fail to effectively enhance the local blood supply (Sanada et al., 2015; Ylä-Herttuala and Baker, 2017). Hence, better understanding of the physiological processes governing the adaptation to ischemia is expected to open new therapeutic avenues to support regain of tissue function.

Mechanisms of vascular repair following ischemia

Acute vessel occlusion and onset of ischemia trigger a prompt endogenous vascular repair program that aims at restoring functional tissue perfusion. This response includes neovessel formation and the opening of collateral arterioles to halt tissue damage and allow for healing and functional restoration (Limbourg et al., 2009).

Vessel occlusion causes changes in fluid pressure that redirect the blood into pre–existing collateral arterioles to bypass the blockade. As blood enters the previously bared collateral vessels, its flow generates shear stress forces at
the vessel wall, triggering a cascade of signaling events that induce local collateral growth (Figure 5b). This way, the blood supply to downstream tissues is increased (Silvestre et al., 2013).

Figure 5. Mechanisms of vascular repair following ischemia. A) The mature and quiescent vasculature in healthy tissues is perfused and lined by mural cells (green in the image) that chaperone the vessels so that they are stable. B) Arterial obstruction (yellow clot) generates shear stress forces that result in the opening of collateral arteries (collateral growth; 1) in the vicinity of the occlusion. C) These collateral arteries redirect the blood flow to the downstream hypoxic tissue. The hypoxic response that occurs in the downstream tissue induces angiogenesis (2, sprouting angiogenesis; 3, intussusception), which results in prompt formation of immature vessels and capillary enlargement (4). For proper functioning, new vessels need to mature and become stabilized by mural cells (5) (Armulik et al., 2011; Potente and Mäkinen, 2017).

In parallel, an angiogenic response driven by local tissue hypoxia (the decrease in the partial pressure of oxygen) takes place (Figure 5c) (Silvestre et al., 2013). Angiogenesis is the formation of neo–vessels from pre–existing ones and is a step–wise process regulated by a plethora of signaling cascades. Initially, the lack of oxygen results in the stabilization of Hypoxia–inducible factor 1 (HIF–1), which escapes proteasomal degradation and, in turn, induces the expression of angiogenic cytokines and growth factors. Among them, VEGF is the most studied one and promotes the formation of new vessels by inducing endothelial cell proliferation, migration and sprouting (Simons et al., 2016). Angiogenesis begins with a marked increase in vessel permeability, where after extracellular matrix remodeling allows the migration of activated endothelial cells that enter the surrounding tissue to form new vessel sprouts that subsequently develop lumen. Driven by strong metabolic demands, an overshooting angiogenic response is believed to result in the formation of excessive vessels, which are initially immature and leaky. Vessel stabilization and maturation relies on recruitment of mural cells, which is dependent on endothelial PDGFβ (Armulik et al., 2011). Thereafter, the vessels specialize and undergo remodeling to develop into a quiescent and hierarchical vascular tree adapted to the tissue (Potente and Mäkinen, 2017).

As part of vascular remodeling, vessel regression involves the removal of superfluous vessels and, guided by hemodynamic forces derived from inadequate blood perfusion, contributes to vascular normalization (Franco et al.,
It has been well-studied during development, for instance in the murine retina, but it has also been described in the adult during luteolysis, when the vasculature undergoes complete and cyclical regression (Modlich et al., 1996), as well as during mammary involution after lactation (Andres and Djonov, 2010). The processes driving vessel regression seem to differ depending on the vascular bed. For instance, withdrawal of survival factors marks vessel regression during the development of the retina in mice (Alon et al., 1995). Interestingly, macrophages have been shown to participate to vessel regression by phagocytosing and clearing endothelial cells during regression of the hyaloid vessels (Mitchell et al., 1998) and during regression of the corpus luteum (Thiruchelvam et al., 2013). In addition, Tie–2 positive macrophages induce endothelial cell apoptosis by production of WNT7B and this way induce vessel regression during embryonic development of the hyaloid vasculature (Rao et al., 2007).

Indeed, ischemic injuries elicit a dynamic and fine-tuned sterile inflammatory response that contributes to restoration of tissue homeostasis and changes as healing progresses. Hypoxia induces the prompt recruitment of neutrophils, monocytes and the activation of tissue–resident macrophages that orchestrate the different phases of repair. Indeed, we discovered a previously unknown subset of neutrophils with pro–angiogenic capabilities (Christoffersson et al., 2010, 2012, 2017; Massena et al., 2015). This neutrophil subpopulation is found in the circulation of both mice and humans, they express higher levels of CD49d, VEGFR1 and C–X–C Motif Chemokine Receptor 4 (CXCR4), and are specifically recruited by VEGF–A to sites of hypoxia. There, they promote angiogenesis and deliver high amounts of MMP–9. In the mouse model of hindlimb ischemia, where the femoral artery is ligated and excised rendering the tissue downstream ischemic, macrophages have been found to support both arteriogenesis (Heil et al., 2006; Schaper et al., 1976) and angiogenesis (Corliss et al., 2016; Sunderkötter et al., 1994), in addition to promoting the regeneration of the muscle fibers (Pimorady-Esfahani et al., 1997; Tidball, 2005). In the present thesis, I study whether macrophages take on novel functions at sites of ischemia in order to improve blood flow restoration during healing using the murine model of hindlimb ischemia (Studies II–IV).
Aims

*It is possible to state as a general principle that the mesodermic phagocytes, which originally (as in the sponges of our days) acted as digestive cells, retained their role to absorb the dead or weakened parts of the organism as much as different foreign intruders.*

Ilya Mechnikov

The overall aim with the present investigation was to characterize how macrophages participate to the maintenance of tissue homeostasis during islet development and during healing of ischemic injury.

The aim of **Study I** was to understand whether macrophages support postnatal development of the insulin–producing β cell population in the pancreas and whether neonatal infections interfered with this process.

Based on the initial observation that macrophages accumulate at perivascular positions in response to muscle ischemia, studies II–IV examine unique functions of macrophages for the restitution of functional tissue perfusion and vascular normalization following ischemic injury. More specifically:

**Study II** aimed to understand whether perivascular macrophages regulate blood flow at sites of ischemic injury, as well as the underlying mechanisms;

**Study III** investigated the fate of macrophages and their contributions to blood vessel maturation following ischemic injury;

**Study IV** characterized the contributions of macrophages to normalization of the newly established vascular network following ischemic injury.
Present Investigation

... we must assume the existence of a living chain that links the particulate stimulus with the blood vessel. This chain will enable a reaction of blood phagocytes even if these are far away from the inflammatory stimulus...

Ilya Mechnikov

The work presented in Study I in this thesis recognizes a new facet of tissue–resident macrophages in supporting the postnatal development of mature insulin producing β cells in the pancreas.

Macrophages accumulate in the neonatal pancreas

We initially observed that macrophages were present in high densities in the neonatal pancreas (two–day–old mice) and regressed to levels comparable to those in adult pancreas prior to weaning (three–week–old mice; Figure 6a). This time of postnatal pancreas development is critical for acquisition of an adult insulin–producing cell mass through β cell functional maturation and β cell mass expansion (Benitez et al., 2012; Bonner-Weir et al., 2016). Thereby, we wondered if macrophages played a role in such processes. In support of this hypothesis, macrophages have been previously shown to promote β cell proliferation and function in adult pancreas by releasing TGFβ, epidermal growth factor (EGF) (Xiao et al., 2014) and IGF–1 (Nackiewicz et al., 2018). In addition, major histocompatibility complex class II (MHCII)–expressing macrophages stimulate β cell proliferation in a PDGF–dependent manner (Ying et al., 2019). Here, we found that MHCII macrophages are present in high density in neonatal murine pancreas (Figure 6b).

In parallel to the expansion of β cell mass, a wave of β cell apoptosis occurs during the postnatal development of the pancreas (Benitez et al., 2012; Bonner-Weir et al., 2016). Macrophages are professional phagocytes, well known for scavenging dying cells during development and injury (Peiseler and Kubes, 2018). Interestingly, we observed that neonatal pancreas harbors a population of phagocytosis–prone macrophages (Figure 6c).
Early–in–life infections interfere with the population of pancreatic macrophages and render mice longstanding glucose intolerant

The immune system is also known to undergo postnatal maturation (Olin et al., 2018) and early life exposure to challenges to the immune system is somewhat associated with higher risk of developing certain diseases including asthma (Laforest-Lapointe and Arrieta, 2017) and T1D (Vatanen et al., 2016) later in life. We therefore postulated that bacterial infections in the neonate could have an effect on resident macrophage populations. To this end, we developed a model where *Staphylococcus aureus* (*S. aureus*) was administered orally to mice twice during the first week of life (10⁷ CFUs; Figure 7a). *S. aureus* administration resulted in transient bacteremia (Figure 7b) and concomitant splenomegaly (Figure 7c), from which mice recovered by week six of age (Figure 7d, e). This intervention did not affect body or pancreas weights at one week of age (Figure 7f) but resulted in a transient reduction of the numbers of pancreatic macrophages, which was normalized by week six (Figure 7g). Subsequent analysis of glucose handling via glucose tolerance test (GTT; 2.5 g/kg intra venous glucose) at week three and six of age –when the mice were no longer infected and pancreatic macrophage levels had normalized– revealed that mice that had been exposed to infections during the first week of life became longstanding glucose intolerant (Figure 7h–k). Interestingly, these mice presented reduced pancreas weights (shown as % of body weight, BW; week three and six of age; Figure 7l, m), which is indicative of a defective postnatal pancreas growth.
Figure 7. Mice exposed to neonatal infections develop longstanding glucose intolerance. A) Schematic representation of the experimental outline of the neonatal S. aureus infection model employed. B) Bacteremia (presence of luminescent S. aureus Xen29 in blood cultures is marked by yellow boxes) was found at week one of age in all mice treated with S. aureus. C) Spleen weight (% BW; n=11–12), D) Presence or absence (yellow or white boxes, respectively, n=7) of luminescent S. aureus Xen29 in blood cultures from mice treated orally with saline or S. aureus (6 weeks following per oral treatment). E) Spleen weight of six–week–old mice (% BW; n=7). F) Body and pancreas weight (% BW) of one–week–old mice (n=11–12). G) Flow cytometry analysis (CytoFlex S) of the pancreas resident macrophage population (CD45+ CD11b+ F4/80+ cells) at week one of age of mice treated with saline or S. aureus during their first week of life (n= 5–6). The numbers of pancreatic macrophages are restored at week six of age (n=2–3). Plasma glucose levels during the course of a GTT (i.v.) of H) three– (n=13–14) and I) six– (n=9–10) week–old mice exposed to neonatal infections. Area under the curve for the GTT at J) week three (n=15–17) and K) at week six (n=11) of life. Pancreas weight (% BW) of L) three (n=3–5) and M) six–week–old (n=8–10) mice of saline– or S. aureus–treated mice. Data shown as average ± SEM; Mann–Whitney U test (C–G and L–M); two–tailed unpaired T Test (H–K and body weight in G); ns: not significant.

Neonatal infections impair β cell development

By immunohistological examination of the pancreas, we discovered that neonatal S. aureus infections interfered with appropriate islet maturation, since decreased intra–islet insulin content (insulin mean fluorescent intensity, MFI; Figure 8a), reduced percentage of proliferating β cells (Ki67 positive; Figure 8b) and reduced percentage of MafA–expressing β cells (Figure 8c) were observed in six–week–old mice that had been exposed to neonatal infections. Further, no changes were detected in the proportion of β cells per islet (Figure 8d), or in islet morphology (Figure 8e). An increase in islet size, islet hyperplasia, which has previously been associated with diabetes, was detected in mice subjected to infections early in life (Figure 8f). Together our data indicate that neonatal infections restrict postnatal β cell maturation.
Figure 8. Neonatal infections affect islet maturation. A) Islet insulin content analyzed as insulin MFI (N=6; n=292–382) and B) percentage of proliferating β cells (N=6; n=292-382) per islet from immunohistochemically examination of sections of pancreas from six–week–old mice. C) Percentage of MafA–expressing β cells presented as average per mice (N=3; n=35–65). D) Percentage of β cells per islet (N=6). E) Islet circularity per mice (N=6; n=292–382). F) Islet area (µm²; N=6; n=292–382). For each mouse, 5–6 pancreatic sections separated at least 200 µm were analyzed. Sections imaged using LSM700, Zeiss and analyzed with ImageJ. Data shown as average ± SEM; two–tailed unpaired T Test (A, E, F); Mann–Whitney U test (B–D). N= number of pancreas analyzed; n= number of islets analyzed.

Neonatal depletion of macrophages recapitulates the effects of neonatal *S. aureus* infections

In an effort to understand whether the effects of neonatal infections were due to macrophage scarcity during postnatal islet maturation, we developed a protocol for transient clodronate–dependent macrophage depletion. Clodronate liposome administration during the first week of life (*i.p.*; Figure 9a) resulted in 86% depletion of pancreatic macrophages at week one (Figure 9b). At week three, the levels of pancreatic macrophages were restored (Figure 9b). Assessment of glucose handling by GTT revealed that mice exposed to transient neonatal macrophage depletion developed defective ability to metabolize glucose, even after the levels of pancreatic macrophages were normalized, at week three and six of age (Figure 9c, d). In consistency with our previous observations, transient neonatal macrophage depletion resulted in reduced pancreas weights (% BW; Figure 9e) and β cell proliferation (Figure 9f) at week six of age.

Taken together, our data in Study I support a role for macrophages for appropriate postnatal maturation of the islets of Langerhans and show that infections occurring during this period restrict the amounts of resident pancreatic
macrophages, thus leading to impaired islet maturation and longstanding insufficiency of glucose handling.

**Figure 9.** Transient neonatal macrophage depletion results in impaired glucose metabolism later in life. A) Schematic representation of the experimental outline of the model of neonatal macrophage depletion. B) Immunohistochemical analysis of the resident macrophage population (F4/80+ cells) at week one and three of age of mice treated with control or clodronate liposomes (i.p.; 5 mg/ml, 25 µl; Encapsula Nano Sciences) during their first week of life (n=6–7). Plasma glucose levels during the course of a GTT (i.v. glucose bolus, 2.5 g/Kg body weight, Fresenius–Kabi) and the area under the curve of C) three– (n=20–23) and D) six– (n= 17–18) week–old mice subjected to transient macrophage depletion in their neonatal period versus control. E) Pancreas weight (% BW; n=16–21) and F) percentage of proliferating β cells (n= 6) of six–week–old mice neonatally exposed to macrophage depletion. Data shown as average ± SEM; Mann–Whitney U test (B, E, F) and two–tailed unpaired t test (C–D).
In Study II, III and IV, the experimental mouse model of hindlimb ischemia was used to study macrophage functions during healing. This is a widely used model of peripheral ischemic disease where ligation and excision of the femoral artery above the superficial epigastric artery branch results in acute ischemia in the downstream limb. Our studies focus on the gastrocnemius muscle, where a strong hypoxia–driven angiogenic response occurs to halt tissue damage. The present investigations unveil different tasks that macrophages undertake in response to ischemic injury to promote vascular restoration and normalization and therefore, preserve tissue function.

MMR– and iNOS– expressing macrophages accumulate at perivascular positions in ischemic tissue

Macrophages accumulated at sites of ischemic injury (CX3CR1GFP, Figure 10a, b, as well as CD45 F4/80 CX3CR1 macrophages; day 3 and 7 post–ischemia induction), where they were found to attain perivascular positions (<1μm from the vascular lumen; b, c). Further characterization by flow cytometry revealed macrophage expression of MMR (Figure 10d) and of inducible nitric oxide synthase (iNOS; day 7 post–ischemia induction; Figure 10e) in ischemic muscles. Interestingly, the majority of MMR+ macrophages co–expressed iNOS, thus presenting features typical of both pro–inflammatory and restorative macrophages (Figure 10f).
Perivascular macrophages in ischemic muscles are important for limb function

In order to understand the functional importance of the increased macrophage numbers in injured muscles, we depleted macrophages with clodronate liposomes (85% reduction at day 3 post–ischemia induction). Tissue damage following ischemia was dramatically exacerbated when macrophages were depleted, as indicated by increased ischemia score (Figure 10g), requiring termination of the experiment at day 3 post–ischemia induction. Thus, this experiment showed that macrophages have a central role in healing of ischemic injuries.
Figure 11. Blood–flow regulation depends on iNOS in ischemic muscles. A, B) Blood flow regulation was assessed as the hyperemic response (the ability to increase blood supply, which we induced by increasing the local temperature; Δ9.9±0.3°C; n=87) using Laser Doppler flowmetry (perfusion units, PFU; Laser Doppler Flowmetry, PeriFlux 4001 Master, Perimed) in parallel with continuous tracking of the skin temperature. C) Basal blood perfusion and D) the hyperemic response (delta perfusion) in response to heat were reduced in the ischemic limbs (healthy limbs: n=34, ischemic limbs: at 3 (n=7) and 7 (n=5) days post ischemia induction). Vascular resistance was calculated by dividing mean arterial blood pressure (measured in the carotid artery) with registered blood perfusion in muscle. The reduction in resistance during hypemia are shown in E) for healthy and F) ischemic muscles and following inhibition of eNOS (L–NAME) or iNOS (L–NIL) (Sigma Aldrich). Data shown as average ± SEM; One–way ANOVA, Tukey’s post hoc test (C–F) *Indicates difference to control group, # indicates difference to “Ischemic 3d or 7d” group, * and # < 0.05.
To evaluate blood flow dynamics following ischemia, we developed a model that assesses the ability of the tissue to upregulate blood flow (heat-induced hyperemia, induced 10°C local temperature increase, measured by Laser Speckle or Lased Doppler flowmetry; Figure 11a–d). Using this model, we identified that blood flow regulation, which in healthy muscles is dependent on eNOS–derived NO (Figure 11e), shifted to being controlled by iNOS–produced NO (Figure 11f). Since macrophages expressed iNOS in ischemic muscles of both mice and humans, we next asked if they assume the control of blood flow regulation at the sites of injury. In the CX3CR1CreERT2;iNOSflo/flo mouse, tamoxifen treatment induced specific iNOS deficiency in 99.98% ± 0.01 of macrophages. This intervention aggravated tissue damage following ischemia (Figure 12a) and showed that macrophage–derived iNOS was essential for the ability to regulate blood flow in ischemic muscles (Figure 12b, c). Our data thereby identify a previously unknown effector function of macrophages as regulators of blood flow during tissue repair.

**Figure 12.** iNOS–expressing macrophages regulate blood flow in ischemic muscles. A mouse model where iNOS deficiency is induced in CX3CR1+ macrophages was used. A) Ischemic damage was significantly aggravated when macrophages where iNOS efficient. B) Laser Doppler examination of tissue perfusion revealed that, although Macrophage–specific iNOS depletion did not affect basal perfusion (PFUs), C) it impaired the hyperemic response (delta perfusion; in response to a 9.9±0.3 °C temperature increase) (Laser Doppler Flowmetry, PeriFlux 4001 Master, Perimed). Data shown as average ± SEM; One–way ANOVA, Tukey's post hoc test (C–F) *Indicates difference to control group, ** < 0.01.

**Macrophages adopt mural cell–like characteristics following ischemia**

In **Study II–IV**, we observed that the majority of macrophages in ischemic limbs presented elongated shapes and were lining the blood vasculature, therefore attaining a morphology that resembles that of mural cells (Figure 10b and Figure 13a). We further characterized the expression of mural cell markers by
flow cytometry and found that indeed macrophages upregulated the expression of Platelet–derived Growth Factor Receptor Beta (PDGFRβ) and Alpha Smooth Muscle Actin (α–SMA) at the site of ischemia (day 7 post–ischemia induction; Figure 13b).

Figure 13. Macrophages in ischemic muscles adopt mural–cell–like phenotypes. A) Representative confocal intravital image of macrophages in the ischemic hindlimb (day 7) (SP8 confocal microscope, Leica). B) Number of PDGFRβ⁺ (n=7) and of αSMA⁺ (n=4) macrophages in healthy vs day 7 post–ischemia gastrocnemius muscles (CytoFlex S). Data shown as average ± SEM. two–tailed unpaired student’s t–test (A, C).

Based on this observation, we hypothesized that macrophages undergo trans–differentiation into mural cells at sites of ischemic injuries. This can only be addressed using genetic fate mapping and heritable labeling, and macrophages were therefore fate–mapped using the Lin–CX3CR1tdTom mice (Cx3cr1– CreERT2 x R26–TdTomato), where heritable expression of tdTomato by CX3CR1⁺ cells is induced by tamoxifen treatment (Figure 14 and Figure 15a). Using intravital confocal imaging, we confirmed that lineage–traced macrophages (also referred to in the text as tdTomato⁺ cells) attained perivascular positions in ischemic muscles (Figure 15b). In addition, they located within the collagen IV endothelial basement membranes, again providing evidence that they establish mural–like close contacts with the vasculature (day 7 and day 21 post–ischemia induction; Figure 15c). Remarkably, we found that in addition to upregulating expression of PDGFRβ (13% ± 1.5 of tdTomato⁺ cells; day 21 post–ischemia induction; Figure 15d), lineage–traced macrophages downregulated the expression of the myeloid markers CD45 and CX3CR1 (31% ± 5.2 of tdTomato⁺ cells; day 21 post–ischemia induction; Figure 15e), indicating the occurrence of a macrophage–to–mural cell phenotype shift in response to ischemia.
Interestingly, while the PDFGRβ+ lineage–traced macrophage population remained constant after ischemia induction (Figure 15g), PDFGRβ+ lineage–traced macrophages increased in numbers following ischemia (Figure 15f). In addition, the loss of myeloid markers was exclusively detected in PDFGRβ+ lineage–traced macrophages (Figure 15h). The potential of macrophages to trans–differentiate into other cell types has previously been suggested, however only during embryonic development. In particular, Yamazaki et al showed that F4/80+ embryonic myeloid progenitors contribute to the development of the mural cell pool of the skin in mouse embryos (Yamazaki et al., 2017). In addition, Yamamoto et al. have shown the capacity of CD31+F4/80+ macrophages of the CNS to differentiate into NG2/PDGFβ/desmin–expressing cerebrovascular pericytes, early during development (Yamamoto et al., 2017). Here, we report for the first time in adult tissue a macrophage–to–mural cell phenotype shift, which occurs at sites of ischemia. Further characterization of the phenotype shift is key to understanding to what extent macrophages trans–differentiate into mural cells in this context.
Figure 15. Ischemia triggers a macrophage–to–mural cell phenotype shift. A) Schematic representation of the experimental outline (hindlimb ischemia induction: red arrow head; time points analyzed: black arrow heads). B) Representative intravital images of lineage–traced macrophages (day 7 and day 21 after ischemia induction; Leica SP8). C) tdTomato+ cells are found within collagen IV basement membranes in ischemic muscles (LSM700, Zeiss). D) Percentage of tdTomato+ cells that express CD45 and CX3CR1, and E) PDGFRβ over time in ischemic muscles (n=3–8). F) The number of tdTomato+ cells that express PDGFRβ increased over time at sites of ischemic muscle (day 21) while G) the number of tdTomato− cells that did not express PDGFRβ remained constant (n=6). H) Characterization of tdTomato−;PDGFRβ− and tdTomato−;PDGFRβ+ in relation to myeloid markers at different times during ischemia (n=4–10). D–H: CytoFlex S. Data shown as average ± SEM; one–Way ANOVA followed by Tukey’s post–hoc test (D, E); unpaired student’s t test (F–H).

Macrophages adopt mural–cell functions in the ischemic hindlimb

We next asked if, beside to transitioning to mural cell–like phenotypes, macrophages acquired mural cell functions. Mural cells are essential for vascular maturation and homeostasis (Armulik et al., 2011), and we therefore examined whether macrophages provided vessel stability in the context of ischemia. For this purpose, we depleted macrophages in ischemic muscles by administration of clodronate liposomes (locally at day 3 and systemically between days 3–7 post–ischemia induction; 67% ± 8 reduction when compared to limbs treated with control liposomes; Figure 16a). This intervention did not affect vessel density (Figure 16b) but instead interfered with vessel maturation, as indicated by a reduction in the number of perfused vessels (lectin [administered in vivo]:CD31 [ex vivo staining] ratio; Figure 16c) and a tendency to increase in vessel permeability (Figure 16d), with concomitant decline in tissue perfusion (basal; Figure 16e).
Macrophages adopt mural cell function at the ischemic site. A) Number of macrophages in control and clodronate liposome treated ischemic muscles (n=3) (i.v. on day 3, 500 µg; intramuscularly daily between days 3–7 after ischemia, 125 µg; Liposoma Research). B) CD31 positive area (expressed as ratio to average of control liposomes treated group; n=12–15). C) Perfused vessels (% of total vessels) analyzed as CD31⁺ vessels (ex vivo staining) that are stained with lectin (in vivo stained; n=8). D) Vessel leakage measured as extravascular IgG staining (n=3). E) Basal perfusion of ischemic muscles treated with control and clodronate liposomes (PFUs—perfusion units; n=11–13). Data shown as average ± SEM. Two-tailed unpaired t test.

In order to easily visualize newly formed vessels in vivo, we employed a model where avascular islets of Langerhans are transplanted into the abdominal muscle of macrophage− and pericyte−reporter mice (CX3CR1GFP⁺/⁺; NG2dsRed mice, Figure 17a), which allows detection of these cell populations. Transplanted islets are quickly re–vascularized by vessels from the recipient mice (Christoffersson et al., 2017) and gained full mural cell coverage by day 5 post–transplantation (Figure 17b. for representative images of re–vascularized islets with neo–vessels lined with NG2⁺ mural cells). Depletion of macrophages (clodronate liposomes; 92.8% ± 0.1 reduction; Figure 17d and c for representative images), which are otherwise promptly recruited to the site of transplantation (Christoffersson et al., 2017; Figure 17c for representative images), did not disturb vessel densities (Figure 17e). Instead, macrophage depletion resulted in acute mural cell scarcity at the islet vasculature (Figure 17f), again suggesting that macrophages may adopt a mural cell phenotype in the context of ischemia.
Figure 17. Macrophage depletion results in prominent mural cell deficiency around newly formed vessels. A) Schematic representation of the experimental outline of the ischemic model of syngeneic islet transplantation: Isolated islets are transplanted into the abdominal muscle of recipient mice (red arrowhead) to enable in vivo imaging of the newly formed vasculature and the cells recruited to the hypoxic sites (black arrowhead). Control or clodronate liposome administration is indicated by the blue arrowhead (i.v., 500 µg, Encapsula Nano Sciences) B) Representative images of islets 5 days after transplantation, showing re-vascularization and that the newly formed vessels are lined by NG2+ mural cell. C) Representative images of islet transplantation sites of control and macrophage–depleted (clodronate) mice (liposome administration indicated with blue arrowheads in panel). D) Positive CX3CR1, E) CD31 and of F) NG2 areas in islet transplantation sites of control (n=11) and macrophage–depleted (clodronate; n=7) mice expressed as ratio to control liposome treated. Data shown as average ± SEM; two-tailed unpaired t test.
PDGFRβ–signaling guides macrophages to perivascular positions in ischemic muscles

The PDGFB–PDGFRβ signaling axis governs mural cell recruitment to blood vessels during angiogenesis as well as sustains this contact during homeostasis (Armulik et al., 2011). Since macrophages in ischemic muscles expressed PDGFRβ, we next asked if this signaling pathway also mediated the perivascular positioning of macrophages. A regime of daily intramuscular administrations of the PDGFRβ inhibitor CP673,451; (between day 2 to 6 post–ischemia induction; 20 µg) did not interfere with macrophage accumulation at the ischemic site (Figure 18a, b). However, macrophages did not acquire elongated shapes (Figure 18a, c), which resulted in reduced macrophage coverage of blood vessel by 30% (Figure 18d), demonstrating that PDGFRβ is important for their interaction with the vasculature.

Vessel normalization occurs at late phases of ischemic hindlimb healing

In order to further explore macrophage functions in the maturation and normalization of the vasculature following an ischemic injury, we first characterized the functional dynamics of the vascular network in our model of hindlimb
ischemia. The vasculature was initially leaky (day 7 post–ischemia) and progressively returned to normal function by day 21 post–ischemia (Figure 19a). In addition, the density of perfused vessels peaked at day 14 post–ischemia induction and later regressed to levels comparable to those of healthy muscle by day 21 post ischemia (Figure 19b, c).

**Figure 19.** Vascular normalization dynamics following ischemic injury. A) Vessel permeability in gastrocnemius muscles at different time points after ischemia induction quantified as IgG positive area (n= 2–4). B) Perfused vessel density in m. gastrocnemius at different time points after ischemia induction imaged in vivo (quantified as percentage of the image volume covered by vessels stained with i.v.–administered fluorescently labeled SBA–lectin; n= 3). C) Representative intravital confocal Z–projection images (scale bar: 100µm). Data shown as average ± SEM; one–Way ANOVA followed by Tukey’s post–hoc test; * indicates statistical significance, *p<0.05, **p<0.01, ***p<0.0001.
Macrophage command vessel regression at sites of ischemia

To investigate if macrophages participated to the observed process of vessel regression (*Figure 20b, c*), we followed a regime of clodronate liposome administrations between day 14 and 21 post–ischemia induction (locally at day 14 and systemically daily between days 14 and 20 post–ischemia induction; *Figure 20a*). Intravital examination of ischemic muscles at day 21 post–ischemia revealed higher vessel densities when macrophages were depleted (*Figure 20b, c*), indicating their contribution to vessel pruning in this context.

*Figure 20.* Macrophages participate to vessel pruning during vascular normalization following ischemic injury. A) Schematic representation of the experimental layout. B) Perfused vessel densities in control and macrophage–depleted ischemic muscles at day 21 post–ischemia (n=4-5). C) Representative intravital images of day 21 ischemic *m. gastrocnemius* of control and macrophage depleted mice (scale bar: 100µm). Data shown as average ± SEM; Mann–Whitney U test.
The Claudin5–GFP (Cldn5) reporter mouse allows visualization of arteries, arterioles and capillaries (Honkura et al., 2018) and we used this model in an effort to detect macrophages engulfing endothelial cells. Flow cytometry analysis of macrophages from ischemic muscles of Cldn5 mice (day 20 post–ischemia) revealed that 33.9% of the macrophages were marked with GFP, suggesting that they may have ingested Cldn5–expressing endothelial cells (Figure 21a and b for representative FlowSight images). Interestingly, 81% of the GFP+ macrophages expressed MMR, a pan marker for phagocytosis (A–Gonzalez et al., 2017), while only 25% of the GFP– macrophages expressed MMR (Figure 21a), suggesting that phagocytic–prone macrophages had efferocytosed superfluous endothelial cells.

![Flowcytometry analysis of macrophages from ischemic muscles of Cldn5 mice day 20 post–ischemia.](image)

**Figure 21.** Macrophages engulf endothelial cell particles at sites of ischemia. A) Percentage of Cldn5+ macrophages and percentage of Cldn5 positive and negative macrophages that are MMR+ at day 20 after ischemia induction (n=2). B) Representative FlowSight images of endothelial cells (green, Cldn5+), Macrophages (red, F4/80+) and macrophages that have phagocytosed endothelium (double positive) (n = 2, scale bar: 20µm).

Together, the data in Studies II–IV recognize macrophages as directors of a healing cascade activated by ischemic injury and aimed at reestablishing perfusion of injured tissues to limit damage and support tissue healing.

Detailed description of the methods employed can be found in Studies I–IV at the end of this thesis.
Concluding Remarks and Future Perspectives

The animal organism is very complex and for this reason it is often hard to explain in simple concepts the phenomena to be observed.

Ilya Mechnikov

This thesis expands the ample repertoire of skills that macrophages display in vertebrates and, once again, highlights their central function in preserving tissue homeostasis. The work presented uncovers that macrophages promote postnatal islet development: our data support that macrophages are important for newborns to develop adult capacity for optimal glucose metabolism (Study I). In addition, the present work identifies different mechanisms by which macrophages promote blood flow restoration and vascular normalization following ischemic injury, namely regulation of blood perfusion, vascular maturation and vessel pruning (Studies II–IV). Additional scientific questions that entail further exploration are raised, some of which are discussed below.

Based on the observation that pancreatic macrophage density peaks in the neonatal mouse and regresses to adult levels prior to weaning, we hypothesized a role for macrophages in postnatal islet maturation in Study I. We show that infections in the neonate affect islet development by interfering with the population of pancreatic macrophages, which results in long term effects on islet function and glucose homeostasis. Validating the initial observation that pancreatic macrophage frequencies peak early in life also in human tissue samples would allow extrapolation of our hypothesis to humans and provide the basis for translational investigations.

Indeed, in Study I we propose that infections in the neonate may account for inter–individual susceptibility for diabetes. Investigating the association between neonatal infections and diabetes development in humans is, therefore, of highest interest. With this aim, individuals exposed to infections early in life can be identified in national health registries based on hospitalization for neonatal sepsis. These individuals can be then followed up for diabetes onset later in life, which is defined based on diagnosis and prescription of anti–diabetic drugs. If an association between neonatal infections and diabetes susceptibility is proven true, our investigation may open opportunities for early diagnosis and for disease management prior to manifestation.
I am particularly intrigued by the evidence that a significant proportion of β cells undergo apoptosis in parallel to the immediate β cell mass expansion that occurs early in life to accommodate the metabolic demands of the developing organism. Are functionally fit β cells being selected over inefficient ones? Given the scavenging phagocytic proficiency of macrophages, it is more than plausible that they play a role in clearing these apoptotic β cells, but whether they actively recognize unfit β cells and concomitantly trigger their apoptosis is not known. What are the signals from incompetent β cells that macrophages may detect to distinguish them from their functionally suitable counterparts?

Studies II, III and IV together recognize a resourceful action plan that macrophages assemble in response to ischemic injury to guarantee healing and vascular normalization.

In Study II, we found that macrophages accumulate at perivascular positions in ischemic tissues and that the local control of blood flow regulation, which in healthy tissue is provided by NO–producing, eNOS–positive endothelial cells, is assumed by iNOS–expressing macrophages during ischemia. Despite the evidence provided, there are still open questions about the effector cells that mediate vessel diameter dilation to control blood flow in the context of ischemia and it would be interesting to assess if mural cells preserve their ability to respond to iNOS–derived NO during ischemia.

In Study II we provide proof of a possible means to locally target macrophages in ischemic muscle to improve functional restoration of regulated blood flow, which can be expanded in future studies. Proof–of–concept experiments demonstrated that local overexpression of CXCL12 enhances the recruitment of CXCR4– expressing, perivascular macrophages, and thereby results in improved blood flow regulation and tissue function. Ischemic diseases are often associated with underlying metabolic conditions, such as hyperglycemia and dyslipidemia. Our proof–of–concept results validate targeting macrophage effector functions as an effective strategy to improve recovery from ischemic injury and it is of clinical relevance to expand our studies to models of such metabolic conditions.

In Study III, we show that macrophages localize perivascularly and upregulate expression of αSMA and PDGFRβ in ischemic muscles, thereby adopting both anatomical locations and marker expression profiles of mural cells. Utilizing fate mapping strategies, we discovered that a portion of the macrophages at the ischemic site undergo a phenotype shift by which they down–regulate the expression of myeloid cell lineage markers (specifically CD45, CX3CR1, and CD11b) and upregulate the expression of the mural cell marker PDGFRβ. To better assess the extent of the macrophage–to–mural–cell phenotype switch, further characterization of these lineage–traced macrophages is essential. For instance, it would be of interest to perform single cell sequencing of lineage–traced macrophages at later stages of post–ischemic recovery.
and compare their expression profile with that of macrophages and pericytes from healthy muscles (and from additional locations). Electron microscopy analysis of the location of PDGFRβ expressing lineage–traced macrophage in relation to endothelial cell basement membranes in ischemic muscles would also provide relevant evidence for this purpose.

We also found that PDGFRβ signaling is important for ischemia–driven macrophage recruitment to blood vessels. Characterization of the molecular effectors that orchestrate the macrophage–to–mural cell phenotype switch remains to be further explored. In Study II, we show that ectopic CXCR4 overexpression increases the numbers and perivascular positioning of macrophages in ischemic muscles, suggesting that this signal contributes to macrophage phenotype switch during ischemia. The corresponding chemokine CXCL12 has previously been shown to withhold bone–marrow–derived cells at perivascular positions (Grunewald et al., 2006). Further, in a mouse model of breast cancer, Arwert et al. showed that CXCL12 is key for the recruitment of tumor associated macrophages to the tumor vasculature, after they have been induced to upregulate CXCR4 expression by tumor–derived TGFβ (Arwert et al., 2018).

To further assess how PDGFRβ signaling contributes to the repertoire of macrophage effector functions during healing of ischemic injuries, it would be suitable to employ a macrophage specific PDGFRβ knock out mouse model. Are PDGFRβ–deficient macrophages, which based on our results would display defective recruitment to perivascular positions, still able to promote vessel functional maturation in ischemic tissues? Or do the vessels instead become more permeable and devoid of mural cells, as observed when macrophages are depleted (Study III)? Also, are PDGFRβ deficient macrophages capable of undergoing the fate shift that we have characterized in Study III?

Our preliminary results in Study IV support that macrophages orchestrate vessel pruning during vascular normalization at late phases of healing of ischemic injuries. This observation needs further investigation. For instance, it would be of interest to evaluate vessel density following ischemic injury in mouse models where macrophage phagocytosis ability is compromised, such as in those lacking functional LXRα and β and PPARγ nuclear receptors or MMR. Moreover, the mechanism behind macrophage–driven vessel regression in the context of ischemia remains unknown. Do apoptotic endothelial cells accumulate in ischemic tissue in the absence of macrophages or when macrophage phagocytic ability is abrogated? Others have shown that macrophages mark endothelial cells to undergo apoptosis during embryonic development of the hyaloid vasculature by secreting WNT7B (Rao et al., 2007). It is therefore of interest to characterize WNT7B expression by macrophages at different time points following ischemia as well as whether vessel pruning is abrogated in genetically engineered mouse models where WNT7B is specifically deleted in macrophages.
As new macrophage functions are discovered, macrophage abilities to fulfill such a vast plethora of tasks–on–demand continue to fascinate the scientific community more than 100 years after Mechnikov proposed his seminal and visionary theory. The work included in this thesis contributed to expanding this theory and suggests that therapeutic enhancement of inherent macrophage functions is attractive for treating ischemic diseases as well as for enhancing β cell maturation and function.

SEGUIRÉ MEDITANDO

Yo me fui para descansar
y elegí un árbol de abultada sombra.

Allí, en una tosca piedra sentado, medité.

Mi cuerpo está tranquilo.
Después de la tarde las estrellas vendrán a tocar las ramas del árbol.

Me sentiré apartado de todo.
Y volveré a sentir que saboreo la vida. Observaré el cielo y sabré si están vivas las estrellas.

No importa la noche para seguir mirando a lo lejos. No importa la oscuridad para seguir pensando.

Me aguarda otra vez el ajetreo de las calles.

Mientras tanto seguiré meditando.

JOAQUÍN HIDALGO
When hearing about the immune system, you probably immediately think about its important role in the defense against bacterial and viral infections. Indeed, the cells of the immune system are spread throughout our body and, like a cellular army, are alert and ready to fight. During my doctoral thesis, I studied a very fascinating cell of the immune system called macrophage. Its name comes from the Greek words *makros*: big and *phagos*: eater. Indeed, macrophages are experts in trapping and ingesting microorganisms in order to kill them by phagocytosis. But I have not studied how macrophages fight intruders. Instead, my doctoral thesis has focused on alternative macrophage functions, which also are necessary for the survival and proper functioning of the individual. Aren't you curious to know more about everything a macrophage is willing to do to help? For me, macrophages are mini-super-heroes and without them, we would not be! Or at least, we would not feel well. Let me explain you a little more...

*Figure 22.* The macrophage is a cell of the immune system and has multiple roles. It is a mini hero that keeps the body healthy, free of infections and diseases.

You might have heard that the only organ in the body where insulin is produced is the pancreas. Specifically, insulin is produced by β cells, which are found in the islets of Langerhans that reside in the pancreas. Insulin is a very important hormone that is necessary for glucose to enter in the cells of the body to be used as a source of energy. When the pancreas does not produce enough insulin, the glucose that cannot enter the cells accumulates in the blood, generating what is called hyperglycemia: very high blood sugar levels. This is the hallmark of diabetes, a harmful chronic disease that affects millions
of people around the world. To avoid the disease, it is important to have a healthy and well-functioning pancreatic $\beta$ cells. When we are born, the $\beta$ cells are immature and need to undergo development to acquire the ability to: 1) effectively monitor blood glucose levels and 2) respond to increased glucose in blood by producing and secreting the amount of insulin needed at any given time. During my PhD, I discovered that macrophages are important for the maturation of $\beta$ cells. If we remove macrophages from the pancreas of mice during their first weeks of life, the $\beta$ cells do not develop properly. Indeed, when these mice are adolescent, they cannot properly control blood glucose levels: we say that they are glucose intolerant. This shows that macrophages are housekeepers of pancreatic postnatal maturation and that disturbing them early in life could predispose individuals to develop diabetes.

![Figure 23. Macrophages aid postnatal pancreas development, which is important or appropriate production of insulin.](image)

During my PhD, I also studied macrophage behavior in ischemic tissues. Ischemia develops when blood flow into a tissue (for example a muscle) is interrupted or reduced, resulting in that enough oxygen cannot reach the tissue, a condition technically known as hypoxia. Without oxygen, the cells cannot generate energy or function properly, the tissue gets damaged and - if blood flow is not promptly restored - the tissue undergoes necrosis and dies. Fortunately, the body is equipped with mechanisms to quickly respond to the lack of oxygen and heal the injury, for example, by inducing the formation of new blood vessels.

In my doctoral thesis, I investigated how macrophages aid the restoration of blood flow in muscle after ischemic damage. Macrophages in ischemic muscles are positioned in close contact with the blood vessels, it looks like if they are hugging them. In the work of this thesis, we discovered that these macrophages assume different functions to help the ischemic muscle heal, namely:

- They control blood flow in the damaged area, which is crucial for the ischemic muscle to heal.
- They change their fate and adopt that of another cell type that is necessary for the proper functioning of new blood vessels.
- They normalize the blood vessel network and remove superfluous blood vessels to restore the tissue after injury.

This thesis has helped unravel the very ambitious and generous action plan that macrophages put together to help heal ischemic damage. I think this is very cool, don’t you?

*Figure 24.* Macrophages aid healing of ischemic injuries so that the injured tissue, in this case the muscle, can recover.

Isn't it fascinating that a cell can accomplish so many tasks? Stay tuned! Science will surely discover more soon.
Si te hablo del sistema inmune, creo que inmediatamente pensarás que este es el sistema que nos proporciona las defensas necesarias para protegernos contra infecciones bacterianas y víricas. De hecho, las células del sistema inmune, que están repartidas por todo nuestro organismo, están siempre alerta, trabajando sin cesar para mantenernos sanos y libres de infecciones. Durante mi tesis doctoral he estudiado una célula del sistema inmune muy particular que se llama macrófago. Su nombre viene del griego *makros*: grande y *phagos*: que come, pues es experta en atrapar e ingerir microorganismos para matarlos; en fagocitarlos. Además de abatir intrusos, los macrófagos desempeñan funciones alternativas que son necesarias para la supervivencia y el funcionamiento adecuado del individuo. Es en el estudio de estas funciones alternativas en lo que se centra esta tesis doctoral. ¿No tienes curiosidad en saber más acerca de todo lo que un macrófago está dispuesto a hacer para ayudar? Para mí, el macrófago es un mini super héroe, y sin él, ¡no sé muy bien que haríamos! Déjame que te explique un poco más…

![Macrófago](image)

*Figure 25.* El macrófago es una célula del sistema immune que cumple muchas funciones diferentes. Es un pequeño héroe que ayuda a mantener el cuerpo sano, libre de infecciones y enfermedades.

Creo que sabes que el único órgano del cuerpo donde se produce insulina es el páncreas. Concretamente, la insulina es producida por las células β, que se encuentran en los islotes de Langerhans que están en el páncreas. La insulina es una hormona muy importante, necesaria para que la glucosa pueda entrar dentro de las células y pueda por tanto ser usada como fuente de energía. Cuando el páncreas no produce suficiente insulina, lo que ocurre en personas con
diabetes, la glucosa que no puede entrar en las células se acumula en la sangre, generando lo que se llama hiperglucemia: niveles muy altos de azúcar en sangre. La diabetes es una enfermedad crónica muy dañina que afecta a millones de personas en el mundo. Para no padecerla es importante conservar el páncreas y las células β sanas. Cuando nacemos, las células β son inmaduras y maduran después de nacer durante el desarrollo posnatal del páncreas, adquiriendo la capacidad de poder monitorear adecuadamente los niveles de glucosa en sangre y de responder a ello segregando la cantidad de insulina necesaria en cada momento. Durante mi doctorado he descubierto que los macrófagos son muy importantes para la maduración de las células β. Si quitamos los macrófagos en ratones durante las primeras semanas de vida, las células β no se terminan de desarrollar correctamente y cuando son adolescentes, los ratones no pueden normalizar de manera correcta los niveles de glucosa en sangre; decimos que son intolerantes a la glucosa. Por tanto, es probable que carecer de macrófagos en el páncreas al inicio de la vida es predisponga para desarrollar diabetes. A partir de ahora, me gustaría explorar esto en humanos.

Por otra parte, en mi tesis también he estudiado el comportamiento de los macrófagos durante isquemia. Isquemia es la condición en la que el flujo sanguíneo a un tejido se ve interrumpido, en la siguiente imagen tenemos al músculo como ejemplo. Sin flujo sanguíneo el oxígeno no consigue llegar al tejido y esto se conoce técnicamente como hipoxia. Sin oxígeno, las células del tejido no pueden generar energía ni funcionar correctamente, el tejido sufre daños y, si no se restablece pronto el flujo sanguíneo (y con ello la concentración de oxígeno), el tejido sufre necrosis y se muere. ¡Pero no te preocupes!, el cuerpo está equipado con mecanismos para responder rápidamente a la falta de oxí-

![Figure 26. Los macrófagos ayudan a que el páncreas se desarrolle después de nacer. Este desarrollo posnatal es muy importante para que el páncreas madure la capacidad de producir insulina de manera apropiada.](image-url)
geno como, por ejemplo, induciendo la formación de nuevos vasos sanguíneos. Y, claro, ¡los macrófagos no iban a ser menos y ahí también van a ayudar!

En mi tesis doctoral he investigado cómo los macrófagos ayudan a restablecer el flujo sanguíneo en el músculo después de un daño isquémico. En el músculo isquémico encontramos a los macrófagos posicionados en contacto con los vasos sanguíneos, parece que los están abrazando. Hemos descubierto que estos macrófagos asumen diferentes funciones para ayudar a que el músculo isquémico se cure:

- Control del flujo sanguíneo en la zona dañada. Sin macrófagos, el músculo isquémico no puede incrementar el flujo sanguíneo cuando lo necesita.
- Cambio de “look”. Parece increíble lo que los macrófagos están dispuestos a hacer. En este caso hemos identificado que algunos macrófagos pierden los “rasgos” que los definen como tal y empiezan a expresar atributos de otras células que son necesarias para que los vasos sanguíneos sean maduros y funcionales en el músculo isquémico. Los macrófagos que asumen el papel y la apariencia de dichas células.
- Normalización de la red de vasos sanguíneos. Para ello, realizan poda de los vasos sanguíneos que son superfluos, toda una obra de arquitectura para quedarse solo con los mejores vasos sanguíneos.

¡Ahora ya sabes que los macrófagos tienen ideado un plan de acción muy ambicioso y generoso para ayudar a sanar daños isquémicos!

Figure 27. Los macrófagos ayudan a sanar los años isquémicos y que el tejido, en este caso el músculo, se recupere.

¿Qué te parece? ¿No es fascinante que una célula pueda llevar a cabo tantas tareas?
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Carmen Herrera Hidalgo,
October 2019, Uppsala
FINAL PREVISTO

He llegado al final de este camino, largo de incidencias, corto de esperanzas, fugaz . . . siempre, que a veces estaba cubierto de yerba, y a veces tan solo hojarascas secas.

Recuerdo el inicio de ilusiones lleno, pensamientos escuetos, promesas y más promesas, grandes rodeos, extraños vericuetos, y el afán de llegar a la meta sin mirar atrás.

Voces amigas que alentaban tu vida sin egoísmos.

Consejos rectos, unas veces, ciertos, otras inciertos.

He llegado al final de este quehacer sin darme cuenta, cuando el cielo está más despejado y limpio, la mente más clara y diáfana y la luz más brillante que nunca.

Pero he llegado al final, al menos, capaz de decir adiós, aunque parezcan mis ojos irritados y mi mente turbada de emociones.

Y pongo punto final, sin duda predispuesto a pedir perdón de cualquier ofensa; ilusionado por tantos recuerdos gratos; y orgulloso de deciros "HASTA LUEGO".

JOAQUÍN HIDALGO
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A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. (Prior to January, 2005, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine”.)