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OXYGEN SENSING IN MYELOID CELLS: IMLICATIONS FOR PHYSIOLOGY AND CANCER

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Oxygen sensing in myeloid cells: Implications for physiology and cancer
THESIS FOR DOCTORAL DEGREE (Ph.D.)

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Utan tvivel är man inte klok" - Tage Danielsson

ABSTRACT

All mammals depend on oxygen in order to produce life-essential energy. They acquire oxygen (O_2) through inspiration. In the lungs, O_2 is gathered by erythrocytes that consequently deliver it to all cells by the cardiovascular system. O_2 levels vary within our bodies. The highest levels are measured in the respiratory system and at lowest in the most peripheral parts of the body, for example skin. Therefore, the physiological levels of O_2 are different in different tissues. During pathological events that result in challenged acquisition of O_2, such as disturbances in the bloodstream or sudden increases of the cell numbers, the need of O_2 often becomes higher than the demand; a condition defined as hypoxia. Because of this, mammals have developed responses that help to manage hypoxia at different levels.

Systemically, low O_2 levels are sensed by chemoreceptors that activate a chain of reflexes leading to more efficient O_2 delivery. Mammalian cells react to hypoxia by increasing O_2 independent energy production, which is done through glycolysis. Cells also activate signaling cascades that stimulate more efficient O_2 delivery in their environment. Cellular reactions to low O_2 are managed by hypoxia inducible factors (HIF), proteins that drive the expression of large amounts of genes necessary for survival in hypoxic environments.

O_2 sensing is important for all mammalian cell types, however it is particularly crucial for motile cells that move through different tissues with a variable O_2 availability. Cells of the immune system need to function also in hypoxic environments. Mononuclear phagocytes (MP) are a group of multifunctional cells of the immune system that are highly dependent on HIF signaling and are also involved in numerous pathological conditions. Previous studies have shown that removal of HIF genes in MP results in dampened disease progression caused by environmental hypoxia, cancer or sepsis. Although the importance of HIF signaling in MP has been shown in several studies, very few of the studies describe the consequences of exaggerated HIF signaling in different states of diseases.

The work in this thesis aims to understand the role of myeloid hypoxia in pulmonary edema, cancer and sepsis, that has been described in three different studies attached to this thesis book. We have shown that augmented hypoxic response in MPs is enough to induce high-altitude pulmonary edema (HAPE)-like symptoms to such extent that it could be used as a model for studying pathological effects of environmental hypoxia in mice. In cancer, on the other hand, we showed that MP hypoxic response contributes to immune suppression by blocking cytotoxic lymphocyte proliferation. The MP-driven inhibition of lymphocyte proliferation occurred during antigen presentation, a process important for lymphocyte activation. Finally, in endotoxin induced sepsis, our studies demonstrated that removal of HIF-1α leads to decreased hypoglycemia due to reduced glycolysis. The strongest reduction in glycolysis was observed in the heart and brown fat.

In conclusion, HIF signaling in MP plays a complex, yet important role in pathogenesis of several major diseases.

SAMMANFATTNING

Livet på jorden är beroende av syre. Däggdjuren använder andningen för att förse sitt blod med syre som sedan förs runt i kroppen med hjälp av hjärt- och kärlsystemet. Genom detta system blir varje cell i vår kropp försedd med syre. Olika delar i kroppen har olika mängder syre. En tumregel är att syremängden är högst i lungorna och lägst i de vävnader som är längst bort från de stora blodbanorna, till exempel huden. I detta avseende är normala syre-mängder relativa och vävnadsspecifika. Hypoxi är en term som används för att beskriva när syre i kroppen eller vävnaden är onormalt lågt. Oftast förorsakas hypoxi av en bakomliggande sjukdom. Sjukdomar som leder till skador i lungorna, blodkärlen eller en onaturlig ökad mängd celler i en vävnad är några exempel på hur vävnader blir hypoxiska.

För många celler i vår kropp är anpassningen till hypoxi livsviktigt. Den cellulära anpassningen styrs genom proteiner som gemensamt kallas för hypoxi-inducerade faktorer (HIF). Denna anpassning är extra viktig för celler som måste röra sig mellan olika vävnader, speciellt för celler som tillhör vårt immunsystem. I denna avhandling studeras en grupp av celler från vårt immunsystem (myeloida celler). Studierna fokuserar på hur dessa celler anpassar sig till hypoxi, samt hur deras anpassning till hypoxi påverkar sjukdomar som lungödem, blodförgiftning samt cancer. Studierna är genomförda med hjälp av genetiska förändringar i myeloida celler, antingen genom att konstant höja nivåerna av HIF, eller genom att ta bort HIF. Avhandlingen är uppdelad i tre olika delar, där den första delen handlar om sjukdomen höghöjdslungödem (HAPE), något som drabbar bergsbestigare. Vanligtvis orsakas HAPE av kärlsammandragning i lungorna med inflammation som en påföljd. Här har våra studier visat att den myeloida hypoxin är tillräcklig för att ge upphov till HAPE-liknande symptom, och vi har utvecklat ett modellsystem för framtida studier om HAPE.

I den andra studien utforskar vi en av mekanismerna som myeloida celler använder för att stoppa det adaptiva immunförsvaret från att bekämpa cancer. Vår forskning visar att myeloid-specifik hypoxi stoppar de cytotoxiska cellerna från att aktiveras.

Slutligen utforskar den sista studien hur det myeloida proteinet HIF-1α påverkar utvecklingen av endotoxininducerad blodförgiftning. Borttagning av HIF-1α i myeloida celler resulterar i minskad endotoxininducerad hypoglykemi samt förbättring av de kliniska symtomen relaterade till blodförgiftning.

LIST OF SCIENTIFIC PAPERS

I. **Gojkovic M**, Darmasaputra GS, Veliça P, Rundqvist H, Johnson RS. *Deregulated hypoxic response in myeloid cells: A model for high-altitude pulmonary oedema (HAPE).* Acta Physiol (Oxf). 2020;229(2):e13461. doi:10.1111/apha.13461

II. **Gojkovic M**, Cunha P, Darmasaputra GS, Rundqvist H, Veliça P and Johnson RS. *Myeloid cell NO prohibits antigen induced cytotoxic T-Cell expansion and promotes melanoma cancer progression in vivo*. Manuscript

III. Fitzpatrick SF, **Gojkovic M**, Macias D, et al. *Glycolytic response to inflammation over time: Role of myeloid HIF-1alpha*. Front Physiol. 2018;9:1624. Published 2018 Nov 22. doi:10.3389/fphys.2018.01624

CONTENTS

LIST OF ABBREVIATIONS

^{18} F-FDG	Fluorodeoxyglucose
APC	Antigen Presenting Cells
Arg	Arginase
ARNT	Aryl Hydrocarbon Receptor Nuclear Translocator
ATP	Adenosine 5′-triphosphate
BAL	Bronchoalveolar Lavage
BMDM	Bone Marrow-Derived Myeloid Cells
C-TAD	C Terminal Transactivation Domain
CBP	CREB-Binding Protein
CCL	Chemokine (C-C Motif) Ligands
CD	Cluster of Differentiation
cGMP	Cyclic Guanosine Monophosphate
COX-2	Cyclooxygenase-2
CSFR1	Colony Stimulating Factor Receptor 1
CXCL	Chemokine (C-X-C Motif) Ligand
DNA	Deoxyribonucleic Acid
eNOS	Endothelial Nitric Oxide Synthetase
FIH	Factor Inhibiting HIF
GFP	Green Fluorescent Protein
GOI	Genes of Interest
HAPE	High Altitude Pulmonary Edema
HIF	Hypoxia Inducible Factors
HLA	Human Leukocyte Antigen
HPV	Hypoxic Pulmonary Vasoconstriction
HRE	Hypoxia Responsive Elements
IFNγ	Interferon Gamma
IL	Interleukin
iNOS	Inducible Nitric Oxide Synthetase
LLC	Lewis Lung Carcinoma
LPS	Lipopolysaccharide
MHC	Major Histocompatibility Complex

MMTV-PyMT	Mouse Mammary Tumor Virus-Polyoma Virus middle T antigen
MP	Mononuclear Phagocyte
MyD88	Myeloid Differentiation Primary Response 88
NO	Nitric Oxide
NOC-18	Diethylenetriamine
O_2	Oxygen
OT-I	Ovalbumin-Specific T-cell receptor
OVA	Ovalbumin
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PD-L1	Programmed Death-Ligand 1
PHD	Prolyl Hydroxylase
RNA	Ribonucleic Acid
SV	Stroke Volume
TLR4	Toll-Like Receptor 4
TME	Tumor Microenvironment
TNFα	Tumor Necrosis Factor Alpha
VEGF	Vascular Endothelial Growth Factor

1 INTRODUCTION: HYPOXIA AND MYELOID CELLS

Oxygen (O_2) levels within the human body are substantially different depending on the tissue. Highest levels are found in inspired air and lowest in our most peripheral tissues. As O_2 is transferred from atmospheric air to the arterial blood, O_2 levels will drop from 21% to 13.2%. The average level of O_2 within tissues is approximately 5% (Figure 1), while in the skin O_2 levels are below 1%. (Carreau et al. 2011)

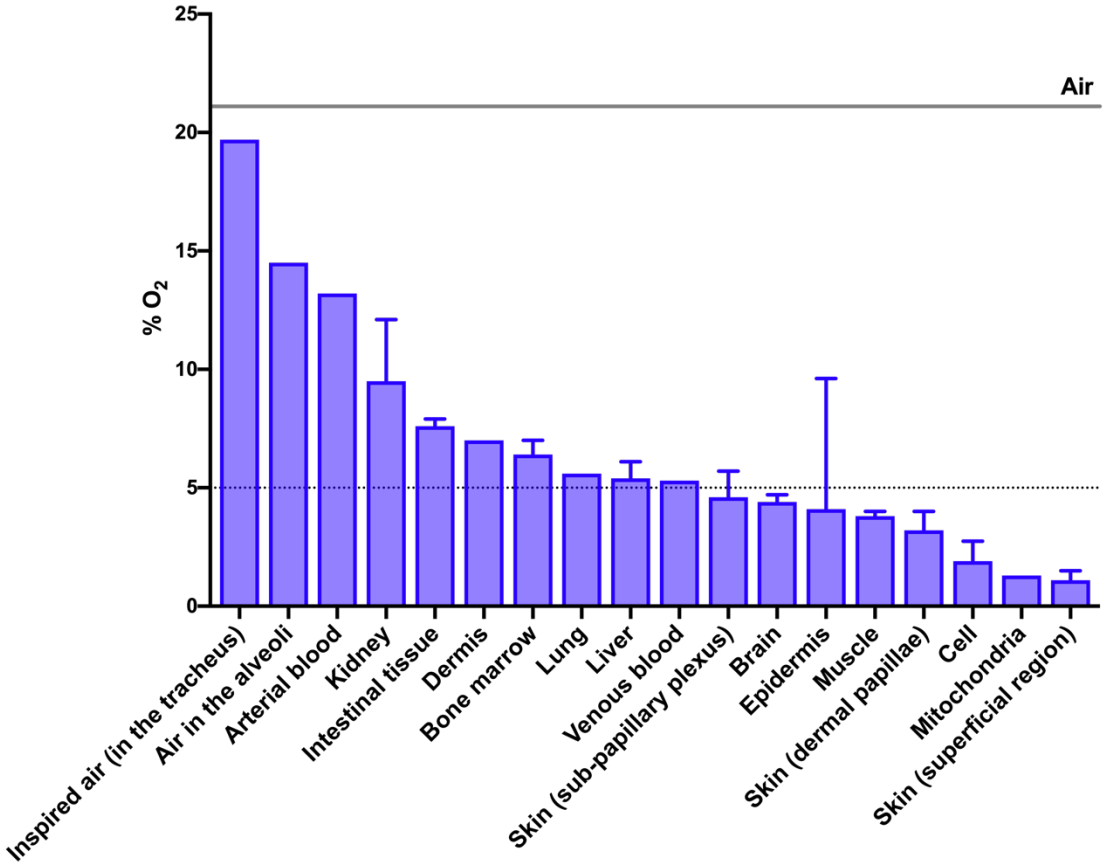

Figure 1. Physiological levels of O_2 are highly variable. Ranked by the highest to the lowest value, average levels of O_2 for each tissue are represented as bar graphs, atmospheric air as a continuous line and the whole-body average as a dotted line. Data collected from Carreau et al. 2011

Abnormally low O_2 levels in tissues or organs defines hypoxia. Hypoxia is often a pathological condition caused by environmental factors (such as high altitude), pulmonary or cardiological pathologies, factors disturbing the blood flow or rapid increase in cellular density in tissues, that are usually caused by neoplastic events or by inflammation (Chao et al. 2009; Schwartz et al. 2011; McKeown 2014). O_2 is vital for mammal life and therefore our bodies have developed complex responses to hypoxia, ranging from systematic reflexes to adaptations at cellular levels (Weir et al. 2005).

1.1 ACUTE RESPONSE TO HYPOXIA

The systemic responses to hypoxia involve a complex signaling network from several chemoreceptor cells. Commonly these signaling pathways aim to compensate for decreased O_2 levels. This is achieved through regulations of pulmonary and vascular functions. O_2 sensing occurs through three major chemoreceptor units localized in the lungs and carotid arteries. (Weir et al. 2005)

In poorly ventilated regions of the lung, alveolar hypoxia leads to regional vascular constriction by the smooth muscle cells, also known as hypoxic pulmonary vasoconstriction (HPV) (Dunham-Snary et al. 2017). This process is meant to shuttle blood from poorly ventilated regions to the more ventilated ones to increase O_2 availability (Dunham-Snary et al. 2017). Thereby, highly ventilated regions of the lung are also well perfused (Sylvester et al. 2012; Baumgardner & Hedenstierna 2016). The regulation of ventilation and perfusion matching is mainly conducted by vascular smooth muscle cells (VSMC) (Aaronson et al. 2005). Alveolar hypoxia initiates inhibition of the potassium channels, which in turn activates voltage gated calcium channels in the cellular membrane of VSMC (Aaronson et al. 2005). The influx of calcium in the cytosol lead to cellular depolarization and VSMC constriction (Aaronson et al. 2005).

As HPV is a local response to hypoxia, pulmonary neuroepithelial bodies initiate cardiorespiratory adjustments to hypoxia (Adriaensen et al. 2002). Airway hypoxia triggers depolarization of neuroepithelial bodies, which in turn stimulate the neurotransmission through the vagus nerve into the central nervous system (Adriaensen et al. 2006). This neurotransmission pathway also controls the heart rate (Ardell et al. 2015).

Furthermore, disturbances in pulmonary ventilation and perfusion leads to decreased levels of arterial O_2 (hypoxemia) (Rodríguez-Roisin & Roca 2005). Here, O_2 sensing occurs in the carotid body, whose main function is to evoke hyperventilation (Lopez-Barneo et al. 2008). The carotid body is located at the branching point between internal and external carotid arteries (Nurse 2014). It consists of mainly two cell types, glomus type I (chemoreceptor cells) and glomus type II (glial-like cells) (Nurse 2014). Decreased levels of the arterial O2 will then trigger depolarization of chemoreceptor cells within the carotid body, leading to their release of neurotransmitters and further neurotransmission (Lopez-Barneo et al. 2008; Nurse 2014).

Collectively, depolarization of O_2 sensitive chemoreceptors trigger events leading to hyperventilation, increased heart rate, venous return and cardiac output in order to increase the delivery of O_2 to the peripheral tissues (Figure 2) (Siebenmann & Lundby 2015; Weir et al. 2005). Where systemic reactions to hypoxia aim to make O_2 delivery more efficient, the cellular response to hypoxia, on the other hand results in transcriptional activities that are essential for both enhanced oxygen delivery (e.g by production of erythropoiesis and angiogenesis) and adaptation to low O_2 (Wilson et al. 2020).

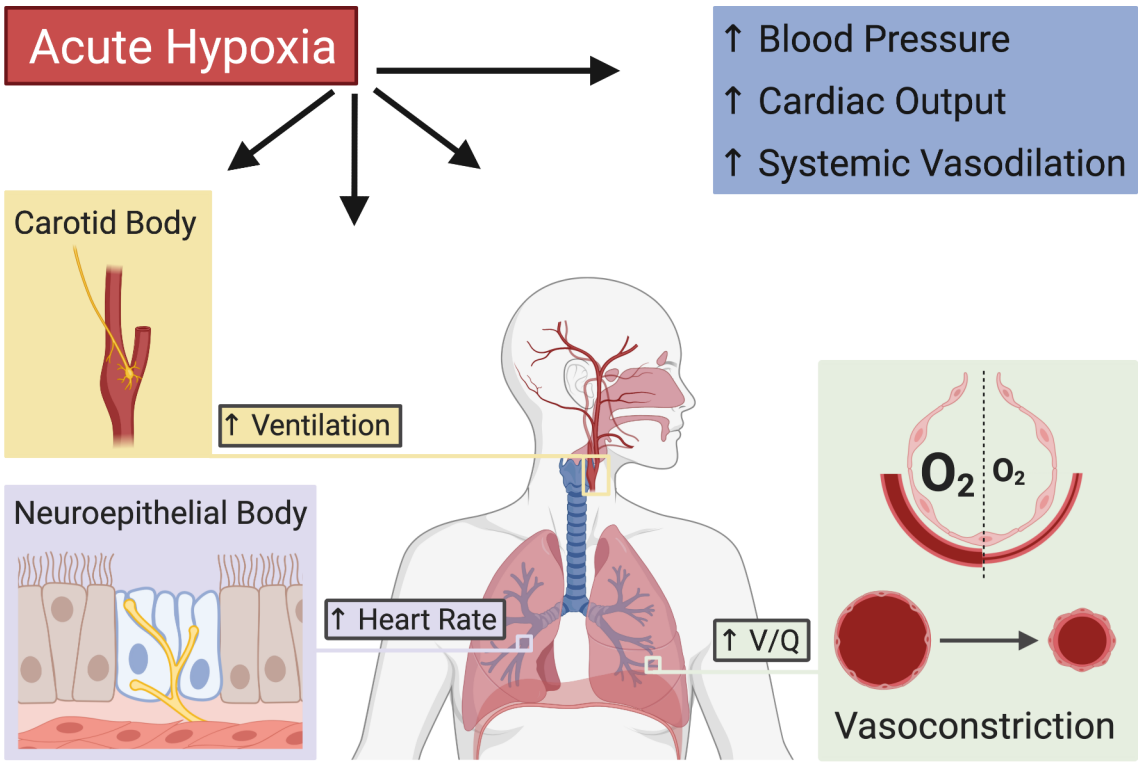

Figure 2: Localization and function of different oxygen sensing chemoreceptors in the human body. V/Q: Ventilation/perfusion ratio.

1.2 HYPOXIA INDUCIBLE FACTORS

The cellular response to O_2 is driven by transcription factors called Hypoxia-Inducible Factors (HIF) (G. Semenza & Wang 1992). HIF consist of 3 alpha subunits (HIF-1α, HIF-2α and HIF-3α) and the beta subunit and its binding partner, aryl hydrocarbon receptor nuclear translocator (ARNT) (Wang et al. 1995). Structurally HIF-1α and HIF-2α contain similar protein domains involved in DNA binding, heterodimerization with ARNT, O_2 dependent degradation, nuclear translocation and coactivator binding domain (Figure 3) (Loboda et al. 2010; Hu et al. 2007). HIF-3α comes in different splice variants with different functions commonly lacking the C terminal transactivation domain (C-TAD) required for binding of chromatin remodeling coactivators P300/CBP (Gu et al. 1998).

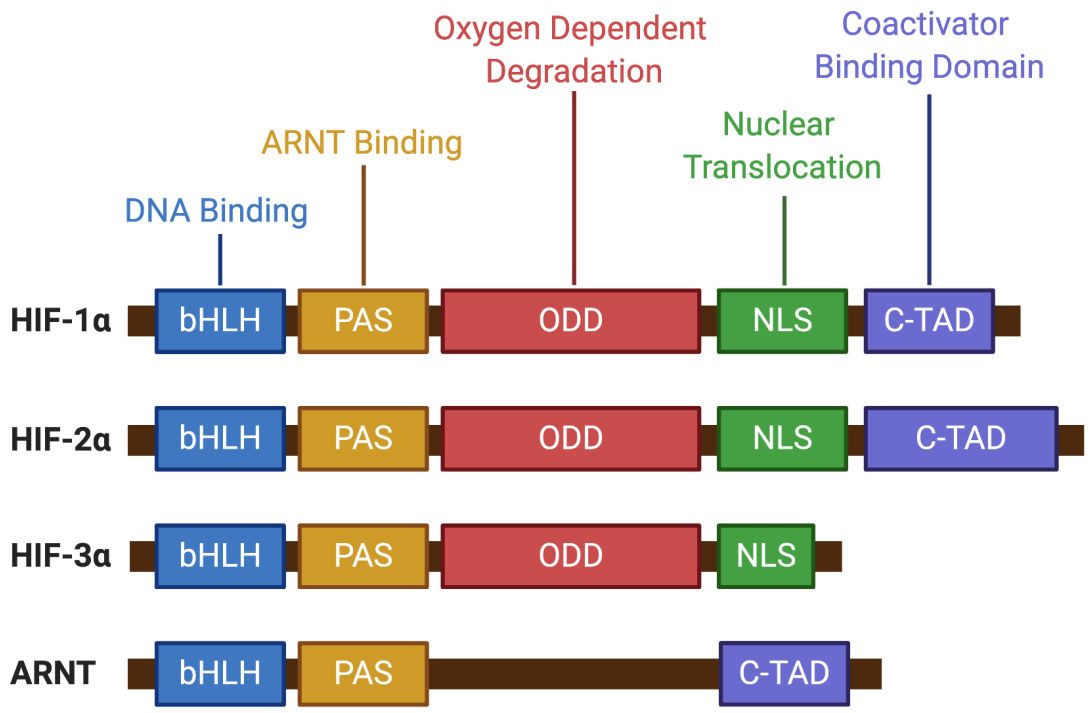

Figure 3: Simplified overview of different protein domains found in HIF-1α, HIF-2α, HIF-3α and the binding partner ARNT. bHLH: Basic helix-loop-helix, PAS: Per-Arnt-Sim, ODD: Oxygen-dependent degradation domain, NLS: Nuclear localization sequence, C-TAD: C-terminal transactivation domain.

HIFs are constitutively expressed (Salceda & Caro 1997) but are tightly regulated post transitionally through two oxygen-dependent pathways. Factor inhibiting HIF (FIH) controls HIF transcriptional activity by hydroxylation of a conserved asparagine residue in the C-TAD domain, therefore preventing the binding of P300/CBP (Lando et al. 2002). HIFs are also constantly degraded through proteasomal activity initiated by hydroxylation of prolines found in the O_2 dependent degradation domain, catalyzed by prolyl hydroxylases (PHDs) (Hon et al. 2002). The hydroxylated prolines are then recognized by the von Hippel-Lindau protein (VHL), which in turn drives HIFs to proteasomal degradation through polyubiquitination

(Maxwell et al. 1999). The catalytic activities of FIH and PHDs are dependent on O_2 as a substrate (Hirsilä et al. 2003; Ehrismann et al. 2006). Decreasing levels of O_2 result in inactivity of FIH and PHDs, allowing HIF to translocate into the nucleus to form a dimer with ARNT (Figure 4). The dimerization results in chromatin remodeling by P300/CBP, DNA binding and initiation of transcriptional activity, through recognition and binding to hypoxia responsive elements (HRE). (Kaelin & Ratcliffe 2008)

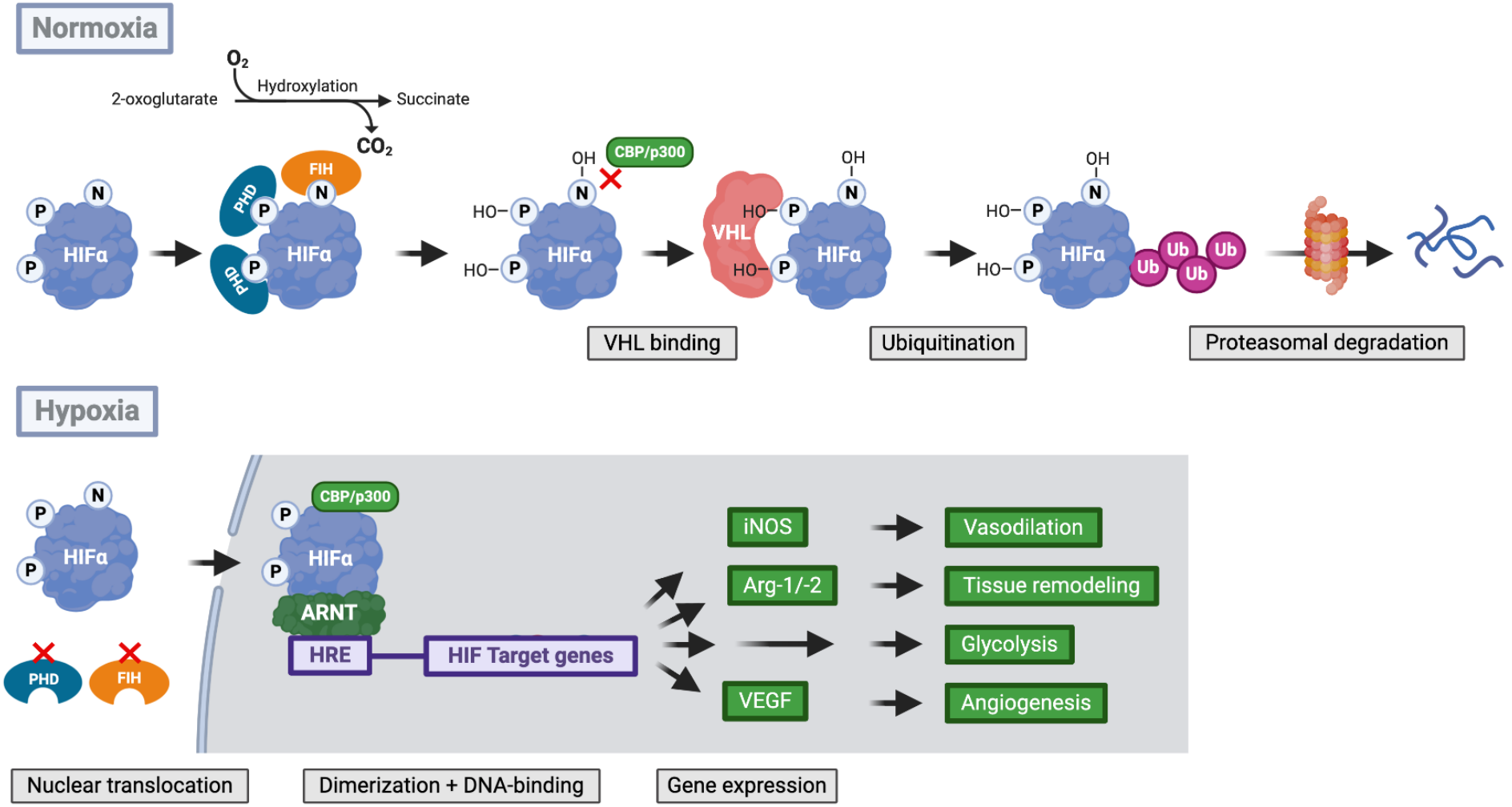

Figure 4: Oxygen (O_2) dependent regulation of HIFs and functional overview. During normoxic conditions HIFs are subjected to proteasomal degradation driven by O_2 dependent hydroxylases. Absence of O_2 results in inactivity of the hydroxylases, stabilization of HIFs and expression of HIF-target genes.

1.3 HYPOXIA SENSITIVE GENES AND ADAPTATION TO CHRONIC HYPOXIA

Transcriptional activity by HIFs is known to induce expression of a broad range of genes involved in angiogenesis, metabolic adaptations, proliferation and inflammation (Cummins et al. 2016; Dengler et al. 2013). HIF-3α consists of several different splice variants, which are known to function as negative regulators of HIF-1α and HIF-2α activity by competing for the binding partner ARNT (Duan 2016). Some studies demonstrate a full transcriptional activity for zebra fish variants of HIF-3α (P. Zhang et al. 2014). However, due to the extensive variation in splice variants between different species, full functional potential of HIF-3α is yet to be explored. HIF-1α and HIF-2α are structurally similar proteins with different roles in the cellular response to hypoxia (Hu et al. 2003; Loboda et al. 2010). In hypoxia, HIF-1α and HIF-2α stabilization is time dependent. Broadly, HIF-1α stabilization peaks within the first 24 hours of hypoxia followed by stabilization of HIF-2α, typically within 24-72 hours in hypoxia (Holmquist-Mengelbier et al. 2006; Reiterer et al. 2019). Time dependent stabilization of the two isoforms reflects the role in biological function; HIF-1α induces transcription of genes important for acute response to hypoxia, while HIF-2α induces expression of genes involved in adaptation to chronic hypoxia (S. A. Patel & Simon 2008).

O_2 is an essential substrate for mitochondrial driven oxidative phosphorylation and its production of the adenosine 5′-triphosphate (ATP) (Nath & Villadsen 2015). Nevertheless, as a cell enters hypoxia, their main source of energy becomes glycolysis, which generates ATP in an O_2 independent manner (Robin et al. 1984). In mammals, upscaling of glycolysis is initiated through HIF-1α activity (Seagroves et al. 2001). To be able to increase the capacity for glycolysis and the ability to produce anaerobic ATP, HIF-1α drives expression of Hexokinases, Phosphoglycerate kinase-1, Fructose-1,6-biphosphatase-1, Pyruvate dehydrogenase kinase-1 and Lactate dehydrogenase A (G. L. Semenza et al. 1994; Maxwell et al. 2001).

Tissue hypoxia leads to increased production of nitric oxide (NO), a signaling molecule that induces vascular dilation (Mil et al. 2002; Casey et al. 2009). NO is produced by the nitric oxide synthase (NOS) from L-arginine (Epstein et al. 1993). The entry of NO into a VSMC leads to activation of guanylyl cyclase, an enzyme that drives production of cyclic guanosine monophosphate (cGMP). cGMP then, in turn, shuts down potassium channels leading to calcium release and VSMC relaxation (Tropea et al. 2018). Signaling of NO occurs in paracrine matter due to its extremely short half time, 0.05-1.8 minutes in blood (X. Liu et al. 1998). Murine HIF-1α induces expression of inducible NOS (iNOS) and therefore increases the cell's capacity to produce NO (Melillo et al. 1995; Palmer et al. 1998). In humans, iNOS and endothelial NOS (eNOS) are the inducible isoforms (Jung et al. 2013; Taylor & Geller 2000), although NO production in humans is known to be driven by hypoxia as well (Levett et al. 2011). However, the direct link between HIF-1α and NOS is yet to be explored. In the endothelial cells, NO is known to induce endothelial cell permeability and their production of vascular endothelial growth factor (VEGF) (Durán et al. 2013; Kimura et al. 2000).

VEGF expression is driven by both HIF-1α and HIF-2α (Forsythe et al. 1996; Flamme et al. 1998), stimulating tissue remodeling by inducing angiogenesis (Nissen et al. 1998) and vascular permeability (Venkatraman & Claesson-Welsh 2019). It also contributes to wound

healing by its anti-inflammatory properties (Lapeyre-Prost et al. 2017). In cancers, VEGF is known to play an important role in tumor metastasis (D. Zhang et al. 2010).

HIF-2α signaling induces expression of factors important for chronic adaptation to hypoxia, such as cell proliferation, erythropoiesis and tissue remodeling (S. A. Patel & Simon 2008). For example, cell proliferation is driven by HIF-2α induction of TGFα expression and c-Myc activity (Zhao et al. 2014; Gordan et al. 2007). To maximize O_2 delivery, HIF-2α promotes erythropoiesis by driving gene expression of erythropoietin (Rankin et al. 2007). Arginase 1 and 2 (Arg-1 and Arg-2) are examples of known HIF-2α targets that contribute to tissue remodeling (Chen et al. 2009; Cowburn et al. 2016; Grasemann et al. 2015; Maarsingh et al. 2011). These enzymes convert L-Arginine to L-ornithine and are found in various tissues and cells (Caldwell et al. 2018). Although involved in many different pathological conditions, these enzymes are known to contribute to hypertension by inhibiting NO production through L-arginine depletion (Huynh et al. 2009; Takeda et al. 2010; Cowburn et al. 2016). Furthermore, this mechanism is also important for wound repair, as decreasing NO is necessary to protect the tissues from damage (Kämpfer et al. 2003).

For example, high-altitude Tibetan populations are reported to have increased peripheral blood flow and NO production (Erzurum et al. 2007), while Arg-2 has been found to be essential for development of hypoxia induced pulmonary hypertension (Cowburn et al. 2016). Overall, HIF-1α and HIF-2α initiate signaling pathways that are important for cellular responses in acute and chronic hypoxia. These examples only mention conditions driven by environmental hypoxia and cardiovascular adaptations to hypoxic situations. However, any motile cells that need to move between different organs and tissues are required to handle rapid changes in O_2 levels (Palazon et al. 2014). Myeloid cells are present in all types of tissues and are extremely motile (Bassler et al. 2019). The need for myeloid cells to move into different environments is also creating the need for them to be able to handle rapid changes in O_2 levels (Bassler et al. 2019), explaining why HIF-1α and HIF-2α are essential factors for their function (Aragones et al. 2011).

1.4 PHAGOCYTOTIC MYELOID CELLS

Cells of the myeloid cell lineage are classically described as cell types that originate from common myeloid progenitors (Weiskopf et al. 2016). These cells consist of megakaryocytes, erythrocytes, granulocytes, monocytes, macrophages and dendritic cells (Weiskopf et al. 2016). Phagocytotic myeloid cells (PMC) are a subgroup within the myeloid lineage responsible for clearance of infected and inflamed tissues, as well as for the the activation of adaptive immune system through antigen presentation and cytokine production (Hünniger & Kurzai 2018; H. Liu & Pope 2004; Grabowska et al. 2018). PMCs include neutrophils, monocytes, macrophages and dendritic cells (Hünniger & Kurzai 2018). Neutrophils have several unique functions and phenotypes, such as being polymorphonuclear, to be first to respond at the site of an infection and the ability to produce neutrophil extracellular traps (Papayannopoulos 2017; Kumar et al. 2018). Macrophages, monocytes and dendritic cells share a significant amount of functional and phenotypic properties, despite differences in origin (Guilliams et al. 2014).

Tissue resident macrophages originate from prenatal stages of development, while circulating monocytes develop from hematopoietic stem cells (Davies et al. 2013). Despite the different origin, monocytes are known to differentiate into inflammatory antigen presenting phagocytes with abilities to promote and inhibit further inflammatory responses (Guilliams et al. 2018). Dendritic cells and macrophages have long been considered as different cell types, however, plasticity of these cells is predominant as their properties vary with their localization and environment (Bassler et al. 2019). Furthermore, phenotypical and functional similarities between these cells complicate their classification (Gottschalk & Kurts 2015). Multidimensional technologies, such as single cell sequencing and cytometry by time-of-flight further contribute to the complexity and challenges of classifications in the myeloid compartment (Günther & Schultze 2019), resulting often in more complex classification (Bassler et al. 2019). Because of this, monocytes, macrophages and dendritic cells will be henceforth referred to as mononuclear phagocytes (MP). Functionally, MPs can be described as a multi-tool cell type (Figure 5). In tissues, MPs function as sentinel cells because of their capability to recognize damage and pathogen associated molecular patterns, furthermore they recruit other cells to the sites of the infection or damage (Stegelmeier et al. 2019; Peiseler & Kubes 2018). In case of infections, MPs clear the infected tissues by phagocytosis and activate the adaptive immunity through antigen presentation and pro inflammatory cytokines production (Kugathasan et al. 2008; Kashem et al. 2017). They also contribute to adaptive immunity by phagocytosing antibody bound pathogens (W. He et al. 2017). As the infection or tissue damage progress into tissue repair, MPs drive this process through secretion of anti-inflammatory cytokines, growth factors (Wynn & Vannella 2016) and clearance of dead cells (Nagata 2018). A broad range of MP functions are dependent on HIF signaling as inflammation and hypoxia are coexisting factors (Palazon et al. 2014).

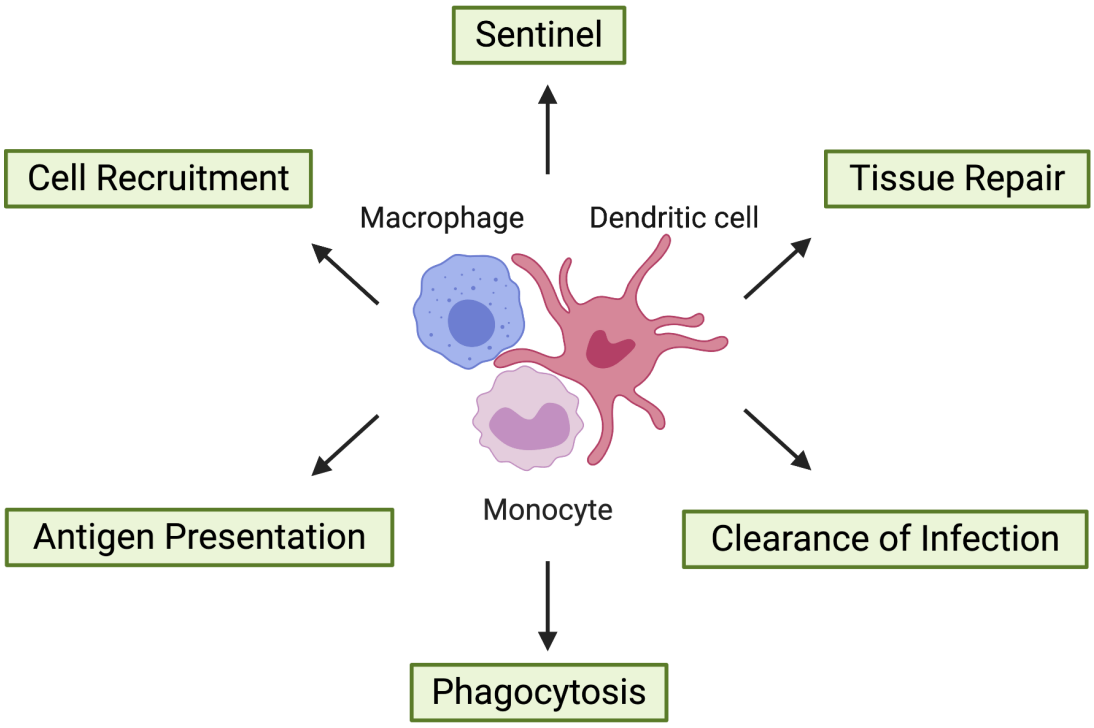

Figure 5: There are several cell types within myeloid phagocytes, however most of these cell types share similar functions.

2 MYELOID CELL HYPOXIA IN DISEASE

The importance of HIF signaling in MP function could best be described by their ability to induce HIF stabilization in an O_2-independent manner. O_2-independent stabilization of HIF-1α in MPs occurs upon recognition of both gram-positive and gram-negative bacteria (Peyssonnaux et al. 2005; Hartmann et al. 2007). HIF-1α stabilization is found to be essential for bactericidal ability of MP, while the deletion of myeloid HIF-1α enhances murine vulnerability to bacterial infections (Peyssonnaux et al. 2005). Earlier studies demonstrated that lipopolysaccharides (LPS), an endotoxin found on the surface of gram-negative bacteria, triggers HIF-1 expression and stabilization in MPs through toll like receptor 4 (TLR4) (Blouin et al. 2003). Besides LPS, the stimulation of MPs by TNFα and IFNγ induces stabilization of HIF-1α, while IL-4 stimulation, results in stabilization of HIF-2α (Albina et al. 2001; Takeda et al. 2010). Furthermore, inflammatory regulated and O_2 independent stabilization of HIF-1α has been found to be triggered by HIF-1α and NF- κB interaction (Rius et al. 2008).

O_2-independent stabilization of HIF during different functions of MP demonstrates an interconnection with HIF signaling. This interconnection plays a significant role in different diseases. For instance, deletion of HIF-1α in myeloid cells reduces clinical symptoms of experimental arthritis due to loss of MPs' migratory ability to the site of inflammation (Cramer et al. 2003). HIF-1α has been suggested to play a role in atherosclerosis, as MPs stabilize HIF-1α upon recognition of a low-density lipoprotein (Shatrov et al. 2003). Acute lung injuries caused by bacterial or parasite infections are attenuated in absence of MP-HIF-1α (Huang et al. 2018; Cahayani et al. 2016). Similar observations have been made in asthma induced tissue remodeling and airway resistance (Alexander et al. 2012; Byrne et al. 2013). In other experimental diseases, complete removal of MP-HIF-1α and HIF-2α signaling deprives the ability to resolve experimental colitis in mice (N. Lin et al. 2018).

2.1 HIGH ALTITUDE PULMONARY EDEMA

Rapid ascent to high altitudes exposes subjects to sudden environmental hypoxia, which in turn will result in extensive HPV and activation of chemoreceptors in the lung and bloodstream (Bandopadhyay & Selvamurthy 2000). These signaling pathways cooperatively trigger hyperventilation, tachycardia, increased cardiac output and pulmonary hypertension (Weir et al. 2005). Prolonged exposure to high altitude induces normalized cardiac output, decreased stroke volume, tachycardia, hyperventilation and development of pulmonary hypertension (Siebenmann & Lundby 2015). Sustained pulmonary hypertension, in turn, leads to an increased extravascular fluid in the lung, advancing to pulmonary edema (Stream & Grissom 2008).

High altitude pulmonary edema (HAPE) is a hypoxia induced noncardiogenic pulmonary edema. Reported incidence of HAPE varies depending on region and population, typically from 0.18% to 5.2% (M Maggiorini et al. 1990; Apte et al. 2014). Although low in incidence,

untreated HAPE may be lethal (Wilkins et al. 2015). HAPE is manifested as shortness of breath, cough and reduced physical activity, tachycardia and moderate fever (Stream & Grissom 2008). The pathogenesis of HAPE is initiated by extensive HPV, not all cases of extensive HPV progress into HAPE (Dehnert et al. 2015).

Several studies revealed genetic polymorphisms associated with a predisposition for HAPE (Luo et al. 2012; Eichstaedt et al. 2020). Some of these genetic variations have been identified in genes encoding for the glucocorticoid receptors and the proinflammatory cytokine IL-6, proposing a role of inflammation in HAPE (Yang et al. 2019; X. He et al. 2018). Transcriptional analysis from HAPE affected persons presented upregulation of genes mainly involved in vascular remodeling and chronic inflammation (Sharma et al. 2014; Yuhong et al. 2018). In addition, inflammation has been demonstrated as a risk factor for HAPE has been demonstrated in early studies (Durmowicz et al. 1997). Some variants of human leukocyte antigen (HLA) were associated with HAPE susceptibility (Hanaoka et al. 1998). Furthermore, increased levels of pro-inflammatory cytokines (e. g. IL-1, IL6 and TNF) and inflammatory cell numbers were found in alveoli of subjects suffering from HAPE (Kubo et al. 1998). Serum levels of MP associated cytokines CCL2 and CCL3, were elevated in individuals with a history of HAPE, these were presumably residues from a chronic inflammation (Mishra et al. 2015). Trials with the anti-inflammatory corticosteroid Dexamethasone were successful in treating HAPE (Marco Maggiorini et al. 2006; Jones et al. 2012). Experimental models of hypoxic pulmonary hypertension confirm the observations found in humans. Rats exposed to chronic hypoxia showed increased pulmonary levels of proinflammatory cytokines: CXCL12, IL6, and CCL2, accompanied by accumulation of MPs at the site of tissue remodeling (Burke et al. 2009). Depletion of MPs in murine models blocked tissue remodeling and development of hypoxic pulmonary hypertension (Vergadi et al. 2011). Genetical removal of inflammatory signal transduction proteins, receptor IL-1R and adaptor MyD88, in myeloid cells damped the progression of hypoxic pulmonary hypertension (Parpaleix et al. 2016). Furthermore, myeloid deletion of HIF-1α results in protection against hypoxia-induced pulmonary tissue remodeling (Kojima et al. 2019).

2.2 CANCER AND METASTASIS

Cancer is the second most common cause of death worldwide. Approximately 17% of global deaths in 2017 were due to cancer (Collaborators et al. 2018). This is a group of diseases initiated by genetic mutations promoting uncontrolled cell proliferation (Fouad & Aanei 2017). The high mutation frequency enables cancer cells to acquire new traits that promote their survival and ability to spread (Fouad & Aanei 2017). In a tumor, cancer cells shape their environment and surrounding cells to form the tumor microenvironment (TME) (Hui & Chen 2015). This process is also known to occur in distant tissues, creating pre-metastatic niches before the arrival of cancer cells (Peinado et al. 2017). Rapidly growing tumor masses result in increased O_2 consumption and underdeveloped blood perfusion, resulting in tumor tissue hypoxia (Muz et al. 2015). Even though cancers are heterogeneous hypoxia is present in most solid cancer types (Figure 6) (McKeown 2014). Another major component of TME are MP

(Kather et al. 2018). Their presence is found in the majority of solid tumors (Kather et al. 2018). Hypoxia and MPs are some of the most important driving forces in TME and cancer progression (A. Patel & Sant 2016). Both of these features are associated with poor prognosis (Walsh et al. 2014; Qian & Pollard 2010).

In murine models of cancer, genetic depletion of MPs did not have any impact on primary tumor growth, but it reduced the metastatic progression of these tumors (E. Y. Lin et al. 2001). Likewise, pharmacological inhibition of colony-stimulating factor receptor 1 (CSFR1) an essential receptor for MP differentiation and proliferation, resulted in reduction of growth and progression in murine models of cervical, breast and prostate cancer as well as glioma (Strachan et al. 2013; Pyonteck et al. 2013; Xu et al. 2013). CSFR1 inhibitors are currently in several clinical trials, where some demonstrated a modest clinical benefit (Cannarile et al. 2017), suggesting a more advanced role of MP in cancer progression.

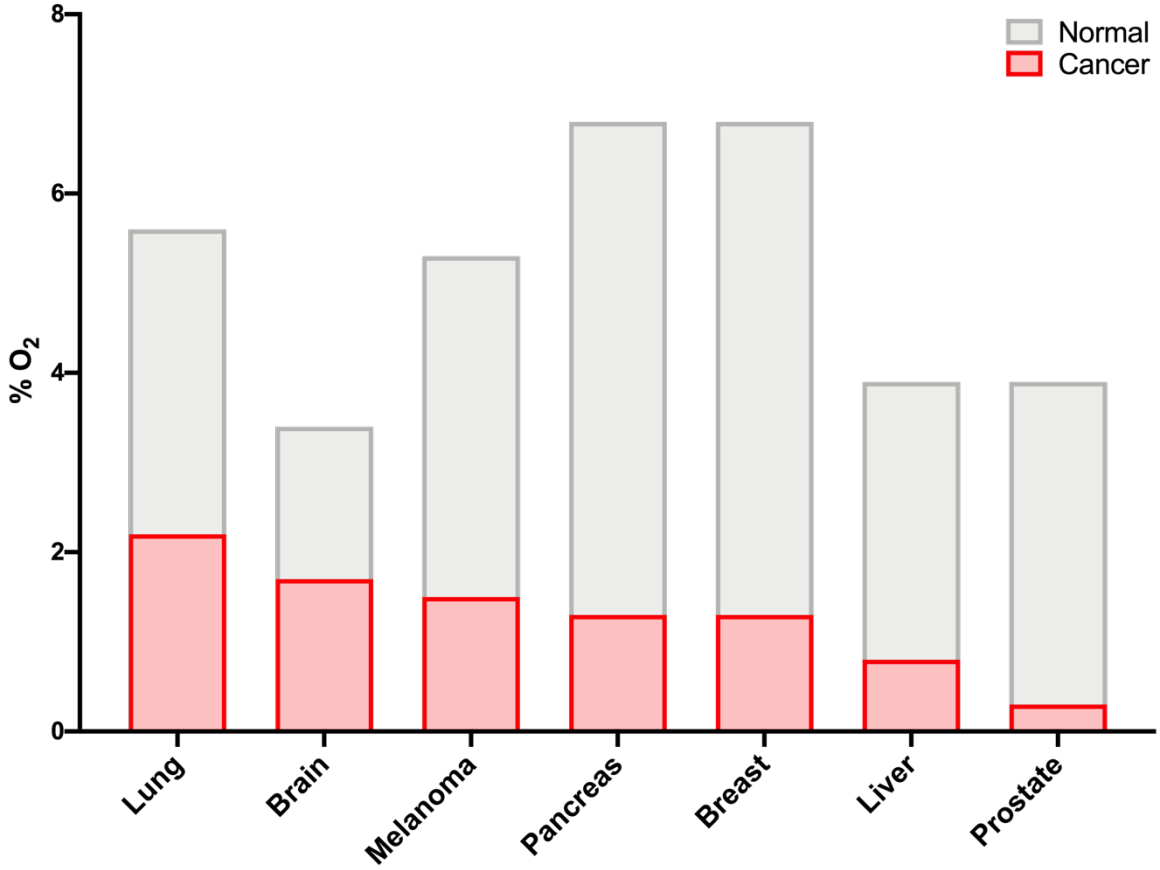

Figure 6: Oxygen levels found in different types of cancer (red) compared to physiological levels in healthy tissue (gray). Data collected from (McKeown 2014)

Myeloid cell HIF signaling plays an important, yet complex, role in cancer. Deletion of myeloid HIF-1α leads to decreased tumor growth in a spontaneous mouse models of breast cancer (Doedens et al. 2010) and to decreased development of obesity-associated liver cancer (Takikawa et al. 2019). Similar observations have been made in chemically induced fibrosarcomas, where mice lacking myeloid HIF-1α are less likely to develop tumors (Henke et al. 2016). However, in other cancer models, such as colitis associated cancer and syngeneic

models of breast cancer, removal of myeloid HIF-2α resulted in reduced tumor formation and growth (Imtiyaz et al. 2010; Susen et al. 2019). Furthermore, in the latter example, deletion of myeloid HIF-1α had no effect on tumor growth (Susen et al. 2019). As benefits in deletion of myeloid HIF-1α and HIF-2α may vary depending on the mouse model, most of the HIF signaling target genes in myeloid cells have shown to contribute to tumor progression (Henze & Mazzone 2016).

CCL2, a cytokine important for recruiting lymphocytes and myeloid cells at the site of infection (Gschwandtner et al. 2019), is induced by hypoxia and HIF-1α signaling (Mojsilovic-Petrovic et al. 2007). Both myeloid cell and cancer cell production of CCL2 is known to drive the tumor progression in all steps; from stimulating tumor growth to driving metastatic spread, by assisting cancer cells in extravasation and intravasation (Lim et al. 2016). Another hypoxia-induced cytokine, IL-6 (Peyssonnaux et al. 2007), is known to protect cancer cells by stimulating cancer cell resistance against chemotherapy (Kumari et al. 2016; Long et al. 2017).

Cyclooxygenase-2 (COX-2) is a driver of cancer promoting inflammation through production of prostaglandins (B. Liu et al. 2015). COX-2 activity promotes tumor immune evasion by regulating both innate and adaptive immune system (B. Liu et al. 2015). HIF-1α activity directly induces COX-2 expression (Kaidi et al. 2006), while COX-2 expression induces both HIF-1α and HIF-2α stabilization (Li et al. 2015; Dong et al. 2018). Inhibition of COX-2 has shown clinical benefits in colorectal cancer (Steinbach et al. 2000) and it is known to improve radiotherapy in other cancers (Salehifar & Hosseinimehr 2016). Although cancer cells express a large amount of COX-2, it has been reported that myeloid cell COX-2 activity can drive up the activity of COX-2 in cancer cells (Li et al. 2015).

Several classical HIF-1α and HIF-2α targets in MPs contribute to angiogenesis, endothelial cell permeability and MP driven immuno-suppression (Figure 7). Removal of myeloid VEGF resulted in increased tumor growth due to normalized vasculature, however, normalized vasculature also made the tumors more sensitive to chemotherapy (Stockmann et al. 2008). In breast cancer models, myeloid deletion of VEGF and anti-VEGF treatment reduces seeding and growth of lung metastasis (Bonapace et al. 2014; Qian et al. 2011). Deletion of Arg-1, a HIF-2α target, reduces tumor growth in a lung cancer isograph model (Colegio et al. 2014). MP HIF-1α drives T-cell suppression through myeloid-secreted NO (Doedens et al. 2010) and increased expression of Programmed death-ligand 1 (PD-L1) (Noman et al. 2014). Increased expression of NOS2 in combination with COX2 is associated with low survival in patients with triple negative breast cancer (Basudhar et al. 2017). Furthermore, targeting iNOS together with immune-checkpoint blockade therapy has been suggested as a potential co-treatment strategy (Ekmekcioglu et al. 2017). Independent of MP, cancer cell HIF-1α is known to directly influence cancer cell antigen presentation by downregulation of Major histocompatibility complex (MHC) Class I (Sethumadhavan et al. 2017).

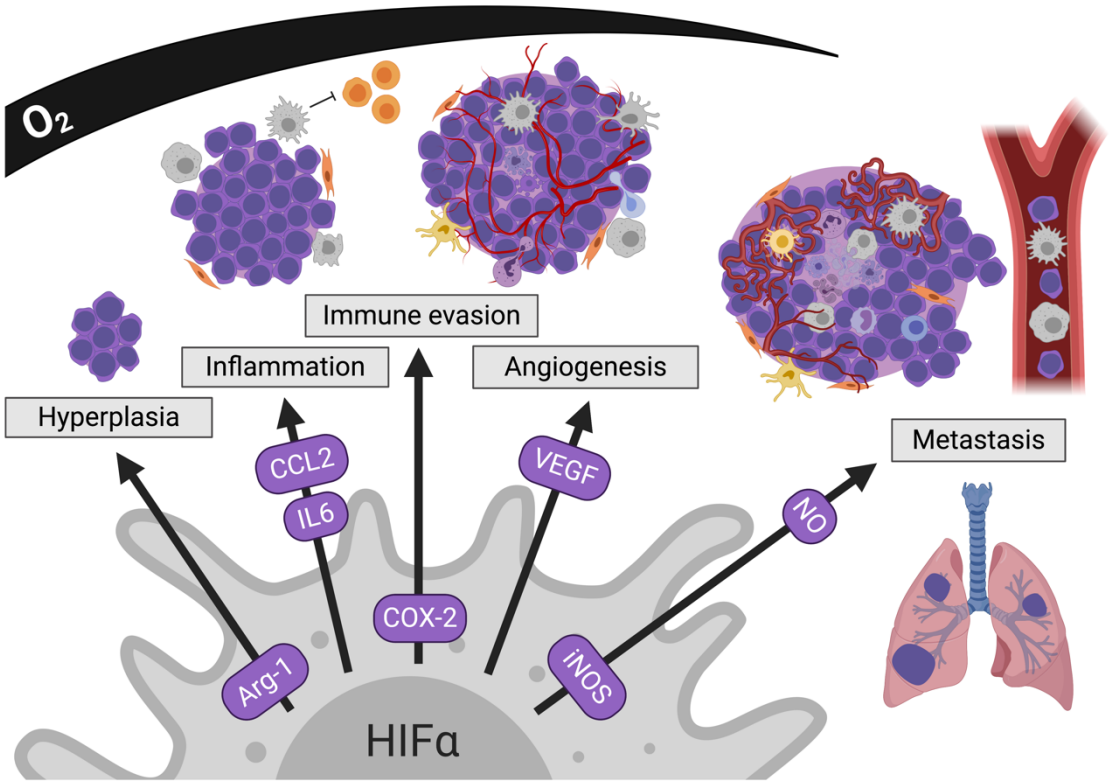

Figure 7: Several direct targets of HIF activity contribute to the major parts of cancer progression.

2.3 SEPSIS

Worldwide, approximately 48.9 million cases of sepsis and 11 million sepsis related deaths have been reported in 2017, this represents approximately 19.7% of total global deaths during the same year (Rudd et al. 2020). Usually, sepsis is caused by an ongoing infection in the respiratory tract (Vincent et al. 2009). Originally, it was separated into four different groups; systemic inflammatory response, sepsis, severe sepsis and septic shock (Lever & Mackenzie 2007), described in order of severity and progression. Current definition of sepsis is divided into two subgroups; sepsis and septic shock. Sepsis is diagnosed by hyperventilation, low blood pressure and decreased consciousness; while septic shock is diagnosed with hypotension, hypoxemia and increased serum lactate levels (Singer et al. 2016). Diastolic dysfunction in patients with severe sepsis is often associated with a high mortality rate (Brown et al. 2012). At this stage, failed perfusion of peripheral organs will lead to multi organ failure (Gotts & Matthay 2016). Besides the ongoing hypoxemia and decreased peripheral perfusion, O_2 consumption and resting metabolic rates are decreasing as the level of severity increases (Kreymann et al. 1993).

In a typical development of sepsis, infectious bacteria will enter the bloodstream causing a cytokine storm by triggering the innate immune system (Stearns-Kurosawa et al. 2011). In most cases where sepsis is caused by gram-negative bacteria (Vincent et al. 2009), the cytokine storm is triggered by MP TLR4 recognition of LPS, localized on the bacterial surface (Jang et al. 2017). Although MPs are the major contributors to the cytokine storm in sepsis (Karakike &

Giamarellos-Bourboulis 2019), their depletion and defects worsen the outcome in sepsis (Lee et al. 2020; Traeger et al. 2008; D. Liu et al. 2018)

The circulatory and respiration defects during sepsis result in tissue hypoxia (Loiacono & Shapiro 2010). In most bacterial infections, HIF-1α has a protective role (Devraj et al. 2017), while in sepsis, HIF-1α may contribute to progression of the disease (Vanderhaeghen et al. 2020), as MP and hypoxia signaling are closely connected. MP recognition of LPS leads to hypoxia independent upregulation of HIF-1α (Nishi et al. 2008; Frede et al. 2006; Peyssonnaux et al. 2007). *In vivo*, myeloid specific deletion of HIF-1α improved survival of mice treated with LPS. Furthermore, serum levels of IL-12 and IL-1β were lower in mice lacking myeloid HIF-1α, compared with wildtype controls (Peyssonnaux et al. 2007). HIF-1α induction of IL-1β was later found to be succinate-dependent (Tannahill et al. 2013). Other studies confirmed the importance of HIF-1α in sepsis. Treatment of mice suffering from LPS or cecal ligation and puncture (release of fecal material into the peritoneal cavity) induced sepsis with 2-methoxyestradiol (a metabolic inhibitor of HIF-1α) enhanced their survival and reduced signs of acute lung injuries (Yeh et al. 2011). Similarly, MP specific HIF-2α knockout mice also showed an increased survival rate, reduction in cytokine production and preserved cardiac function during the LPS challenge (Imtiyaz et al. 2010). Transcriptomic analysis of PBMC from 265 patients suffering from sepsis revealed two types of sepsis response signatures with different mortality rates. The group of patients with a higher mortality rate had higher expression of HIF-1α and HIF-2α (Davenport et al. 2016). After an infection, the immune system enters an immune suppressive stage to protect tissue from further damage and initiate healing; a stage also known as endotoxin tolerance (D. Liu et al. 2018). Endotoxin tolerance followed by sepsis is usually associated with high risks of secondary infections (López-Collazo & Fresno 2013). HIF-1α has been found to contribute to endotoxin tolerance in MP by upregulation of the immunosuppressive surface protein PD-L1 (Avendaño-Ortiz et al. 2017).

3 AIMS

The aim of this thesis is to understand the function and role of myeloid cell hypoxia. This includes understanding the effects on the myeloid functions and their involvement in pulmonary physiology. Furthermore, studies included in this work investigate the role of myeloid hypoxia in major diseases. Specifically, this thesis aims to:

I. Understand the role of myeloid cell hypoxia in pulmonary physiology and their influence on pathological events leading to high altitude pulmonary edema.

II. Deepen the understanding of HIF signaling in myeloid cell-driven activation of adaptive immune-response in cancer and overall influence of deregulated hypoxia signaling in myeloid cells.

III. Gain deeper knowledge on how myeloid HIF-1α contributes to the pathogenesis of sepsis

4 MATERIALS AND METHODS

This section focuses on materials and methods used to perform experiments from the results section "Complementary data" (unpublished data). Detailed information about methods involving paper I, II and III can be found in the respective articles/manuscripts.

4.1 ANIMAL MODELS

Myeloid-specific knockout mice targeting genes involved in hypoxia signaling were used. These models were generated through crossbreeding mice carrying genes of interest flanked with LoxP sites (GOI^{fl}) with transgenic mice expressing Cre recombinase under the lysozyme M promoter (Clausen et al. 1999). Experimental litters consist of phenotypically wild type mice homozygous for flanked genes ($GOI^{fl/fl}$) and myeloid specific knockouts homozygous for flanked genes and hemizygous for Cre ($GOI^{fl/fl}$ $LysM^{Cre}$). Cre recombinase driven by the LysM promoter ($LysM^{Cre}$) have been reported to be efficiently expressed in neutrophils and macrophages, and to a lesser extent in other myeloid cells, such as monocytes, dendritic cells and granulocytes are also affected (Abram et al. 2014).

4.2 CANCER MODELS

The murine model of spontaneous breast cancer was acquired through breeding $VHL^{fl/fl}$ $LysM^{Cre}$ mice with C57/Bl6 mice hemizygous for mammary tumor driven by Polyoma Virus middle T antigen (MMTV-PyMT). The age of onset was determined when the first palpable tumor was detected, while the age of death was determined by the humane endpoint of tumor size equal to 1000 mm^3. At this point tumor and lung tissues were collected for histological assessment of metastasis.

Lewis lung carcinoma (LLC) cell line expressing green fluorescent protein (GFP) were used for orthotopic tumor models (LLC^{GFP}) (Branco-Price et al. 2012). Mice aged 11-13 weeks were injected subcutaneously with 500 000 cells LLC^{GFP}. Tumor size was measured with a caliper three times per week. In order to study the metastatic establishment in our mouse models, 200 000 LLC^{GFP} cells were injected intravenously through the tail vein. Lungs were collected three weeks later. The number of metastasis and the metastatic burden were established through histological assessment.

In order to determine the time dependent dynamics of immunological populations in the lungs during establishment of metastasis, mice were injected with 200 000 LLC^{GFP} intravenously followed by collection of lungs at 2, 7, 14 and 21 days post injection. Lungs were then processed and analyzed by flow cytometry in a similar manner as previously reported (Gojkovic et al. 2020; Fitzpatrick et al. 2018).

4.3 CLODRONATE TREATMENT

LysM^{Cre} mice with Cre dependent tdTomato expression (tdTomato LysM^{Cre}) were injected with liposomes (containing clodronate or PBS) intraperitoneally twice, with a 72 hour gap between the injections. Lungs were perfused, collected and analyzed 24 hours after the last injection. Permeability assay for clodronate treated VHL$^{fl/fl}$ LysM^{Cre} was performed as reported previously (Gojkovic et al. 2020)

4.4 TRANSCRIPTOMICS

Bone marrow-derived myeloid cells (BMDM) were prepared from VHL$^{fl/fl}$ and VHL$^{fl/fl}$ LysM^{Cre} bone marrow as previously reported (Gojkovic et al. 2020). Mature BMDM were then cultured in three different conditions, cultured either in media alone (untreated group), 10 ng/mL TNF and INFγ or 10 ng/mL IL4 and IL13. 48 hours after the treatment, cells were collected and lysed using RNeasy Plus (Qiagen, cat nr: 73404) lysis buffer according to the manufacturer's instruction. Sample lysates were then sent off for further analysis and RNA sequencing. Statistical analysis of acquired transcriptomics data was done with iDEP (Ge et al. 2018).

5 RESULTS AND DISCUSSION

5.1 PAPER I: DEREGULATED HYPOXIA SIGNALING IN PULMONARY EDEMA

Inflammation contributes to pathogenesis of HAPE (Kaminsky et al. 1996; Durmowicz et al. 1997). As described earlier, individuals suffering from HAPE demonstrated increased levels of proinflammatory cytokines in bronchoalveolar lavage (BAL) and blood (Mishra et al. 2015; Kubo et al. 1998). Studies in animals revealed a recruitment of myeloid cells to the pulmonary site in early exposure to hypoxia (Vergadi et al. 2011). Our study demonstrated that myeloid cell hypoxia contributes to an important role in HAPE pathogenesis. Measuring wet and dry lung weights revealed that mice lacking myeloid VHL had increased levels of pulmonary fluid. Signs of endothelial cell fenestration and increased pulmonary permeability in $VHL^{fl/fl}$ $LysM^{Cre}$ mice revealed a spontaneous development of pulmonary edema (Figure 8).

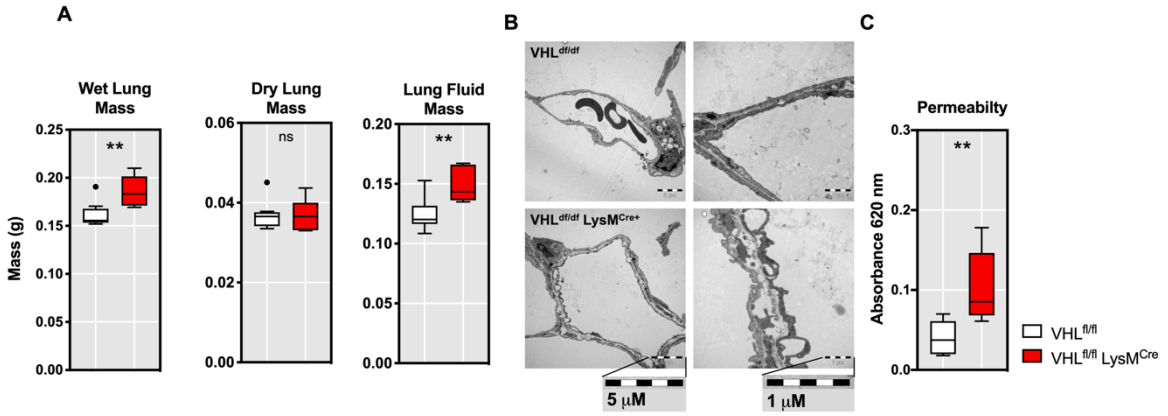

Figure 8: Deletion of myeloid VHL results in pulmonary edema. (A) Total lung weights in grams, dehydrated lung weight and lung fluid mass calculated as a difference between total lung weight and dry weight. (B) Representative electron microscope pictures demonstrating detached endothelial cell layer (endothelial fenestration) in $VHL^{fl/fl}$ $LysM^{Cre}$ mice. (C) Pulmonary endothelial permeability measured by quantification of Evans blue entering alveolar space from the blood stream. Data presented as Tukey boxplots, * $P < 0.05$, ** $P < 0.01$, ns = not significant. Statistical analysis was performed with unpaired T test, n = 7–8 mice per group.

Further on, the immunological characterization of BAL in $VHL^{fl/fl}$ $LysM^{Cre}$ showed a similar profile as described in individuals suffering from HAPE. However, a similar assay performed on the whole lung demonstrated opposite results, showing decreased numbers of alveolar macrophages, where data from BAL had a clear increase in numbers of alveolar macrophages. In the literature, human studies are focused on the components of BAL or blood serum, as pulmonary biopsies are associated with severe risks (Yeow et al. 2004), therefore leaving little data to compare our findings with clinical cases. The transcriptomic data in our study showed an upregulation of several genes that are known to disturb cell-to-cell adhesion, suggesting that increased amount of alveolar macrophages in $VHL^{fl/fl}$ $LysM^{Cre}$ mice caused the difficulties of alveolar macrophages to attach to the surface of the alveolar epithelium.

Reduced physical performance has been reported as a symptom of HAPE (Stream & Grissom 2008). Exposing mice to voluntary running revealed a slight reduction in time spent running. Yet, a strong reduction in covered distance and speed could be observed. Echocardiography in resting mice showed a decreased stroke volume, with unchanged cardiac output. Reduced stroke volume (SV) has previously been observed in HAPE, similar to our observations (Gupta et al. 2016). Reduction of SV could be explained by the increased vascular resistance in the lungs causing a reduced pulmonary venous flow to the heart, explaining why less blood will reach the peripheral tissues in each pulsation of the heart. The reduced peripheral blood flow will result in less O_2 reaching the muscles, which are required for any physical exercise (Figure 9).

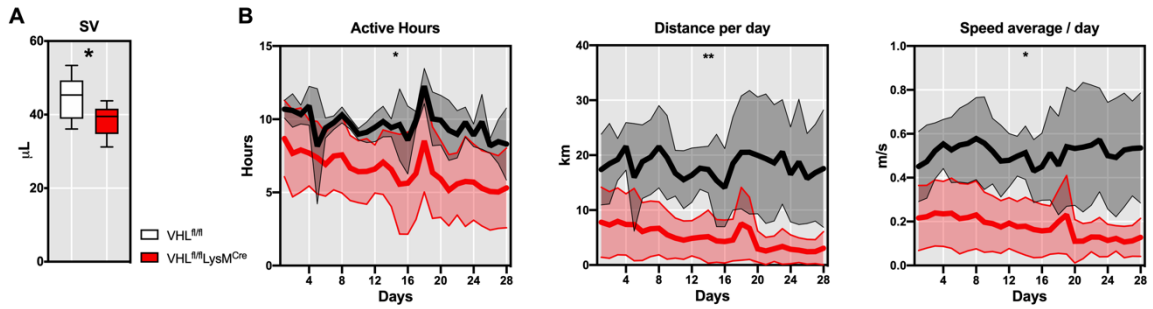

Figure 9: Myeloid hypoxia signaling results in reduced stroke volume and physical performance. (A) Stroke volume measured on resting mice by echocardiography. (B) Mice were housed with running wheels monitoring daily activity, distance and speed of running. Data presented as Tukey boxplots and xy graphs with standard deviation, * $P < 0.05$, **$P < 0.01$. Statistical analysis was performed with unpaired T test, n = 8–10 mice per group, 2 mice/wheel.

Cellular adaptation to hypoxia through HIF-1α and HIF-2α involves transcription of numerous genes (Dengler et al. 2013) and therefore requires a wide approach in understanding the biology of myeloid hypoxia in the pulmonary tissue. Concerning this, we performed RNA-sequencing of whole lungs from $VHL^{fl/fl}$ and $VHL^{fl/fl}$ $LysM^{Cre}$ mice. Deletion of myeloid VHL resulted in transcriptional changes of more than 400 genes. Many of these are known to be involved in endothelial permeability, regulation of blood flow and inflammatory processes, showing a complex contribution of HIF signaling in HAPE (figure 10).

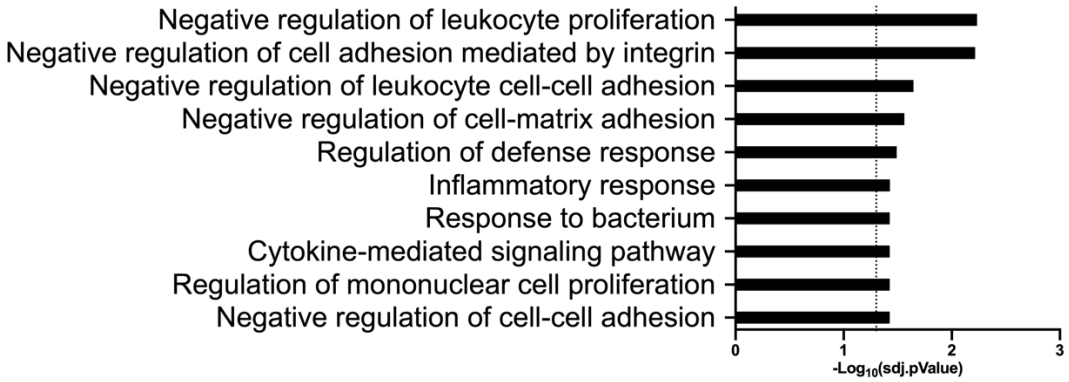

Figure 10: GO pathway enrichment analysis of $VHL^{fl/fl}$ vs $VHL^{fl/fl}$ $LysM^{Cre}$. Adjusted P Value presented as negative Log_{10}.

The environmental hypoxia of high-altitude triggers extensive pulmonary vasoconstriction, causing hypertension and increased vascular permeability, ultimately resulting in pulmonary edema. Simultaneously, alveolar hypoxia will trigger a cellular hypoxic response mediated through HIF activity. Several factors downstream of HIF will drive the inflammatory response, vascular permeability (inflammation itself is known to trigger both vascular permeability and HIF activity) and edema. In a nutshell, both cellular and vascular adaptations to hypoxia will drive events leading to pulmonary edema.

In our mouse model the chain of events leading to pulmonary edema is by myeloid cell HIF signaling. As our data demonstrated, factors driving inflammation, such as receptors Trem2, Cx3cr1 and chemokine Cxcl15 will contribute to events leading to vascular permeability. However, other genes controlled by HIF signaling, such as Spock1, Serpine1 and Mmp12, will cause vascular permeability directly. Bypassing pulmonary vasoconstriction, myeloid hypoxia itself will drive the pathological events similar to those in HAPE (Figure 11).

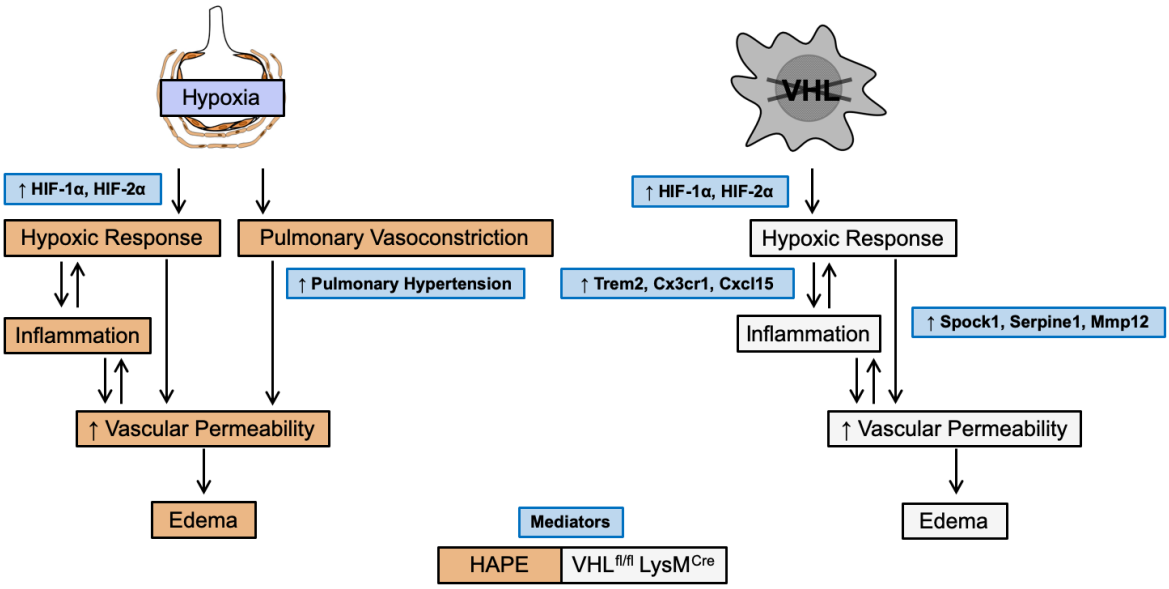

Figure 11: Illustrations of pathological events leading to HAPE in humans vs $VHL^{fl/fl}$ $LysM^{Cre}$ mice with an activated hypoxic response in myeloid cells, causing an $VHL^{fl/fl}$ $LysM^{Cre}$ HAPE-like edema and bypassing the pulmonary vasoconstriction.

5.2 PAPER II: MYELOID NITRIC OXIDE BLOCKS CYTOTOXIC T-CELL PROLIFERATION

Infections and cancers are mainly resolved through antigen recognition of cells from the adaptive immune system. However, in order to activate the adaptive immune response, these cells require antigen presentation through antigen presenting cells (APC). Tissues are constantly cleared from dead cells through myeloid cell phagocytosis. Cellular debris are processed, and parts of proteins presented on the surface of the myeloid cell. (Guermonprez et al. 2002). In cancer, APC activation of cytotoxic CD8 T cells is an essential part of tumor clearance by the immune system (Broz & Krummel 2015). After engulfing cancer cell debris, an APC will present cancer neoantigens, a byproduct of non-synonymous mutations, on its surface through MHC Class I and Class II (Reeves & James 2016). By recognition of neoantigens, presented through MHC Class I, CD8 T cells with a compatible T-cell receptor (TCR) will become activated and mature into a cytotoxic T-cell (Guermonprez et al. 2002), a process accompanied by clonal expansion. Several factors in the tumor microenvironment are known to have a negative impact on APC-dependent CD8 T-cell activation (Fu & Jiang 2018), many of which are driven by hypoxia and HIF signaling (Vaupel & Multhoff 2018).

In order to understand how a hypoxic environment influences the APC ability to activate cytotoxic T cells, we monitored hypoxic antigen presentation in vitro. This was done by activation of BMDMs with LPS, followed by introduction of an MHC class I-restricted antigen (SIINFEKL) of chicken ovalbumin (OVA), and co-cultured with OVA-specific CD8 T Cells (OT-I), as illustrated in Figure 12. The BMDM and OT-I co-culture was then performed under atmospheric oxygen conditions (21% O_2) or hypoxic conditions (5% or 1% O_2)

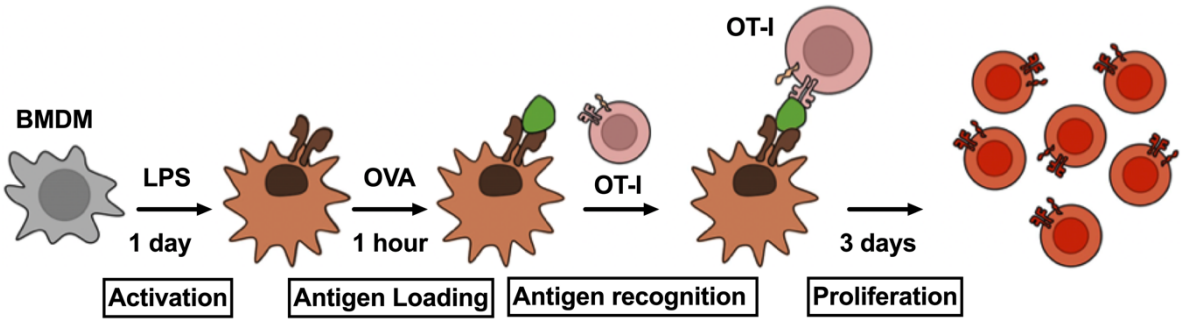

Figure 12: A schematic overview of the antigen presenting assay. Bone marrow myeloid cells (BMDM) were activated with LPS followed by antigen loading and introduction of antigen specific T-cells, for details please see paper II.

Initially, cultures of OT-I cells alone did not demonstrate any changes in ability to divide and proliferate after activation with antigen when cultured in hypoxic conditions. However, when OT-I cells were activated through BMDM antigen presentation, hypoxia blocked their ability to divide and proliferate. While performing the antigen-presenting assay in 21% O_2 with BMDMs lacking VHL resulted in a similar outcome as observed in hypoxic conditions, thus indicating that hypoxia signaling in myeloid cells is responsible for the blockade of T-cell division and proliferation (Figure 13).

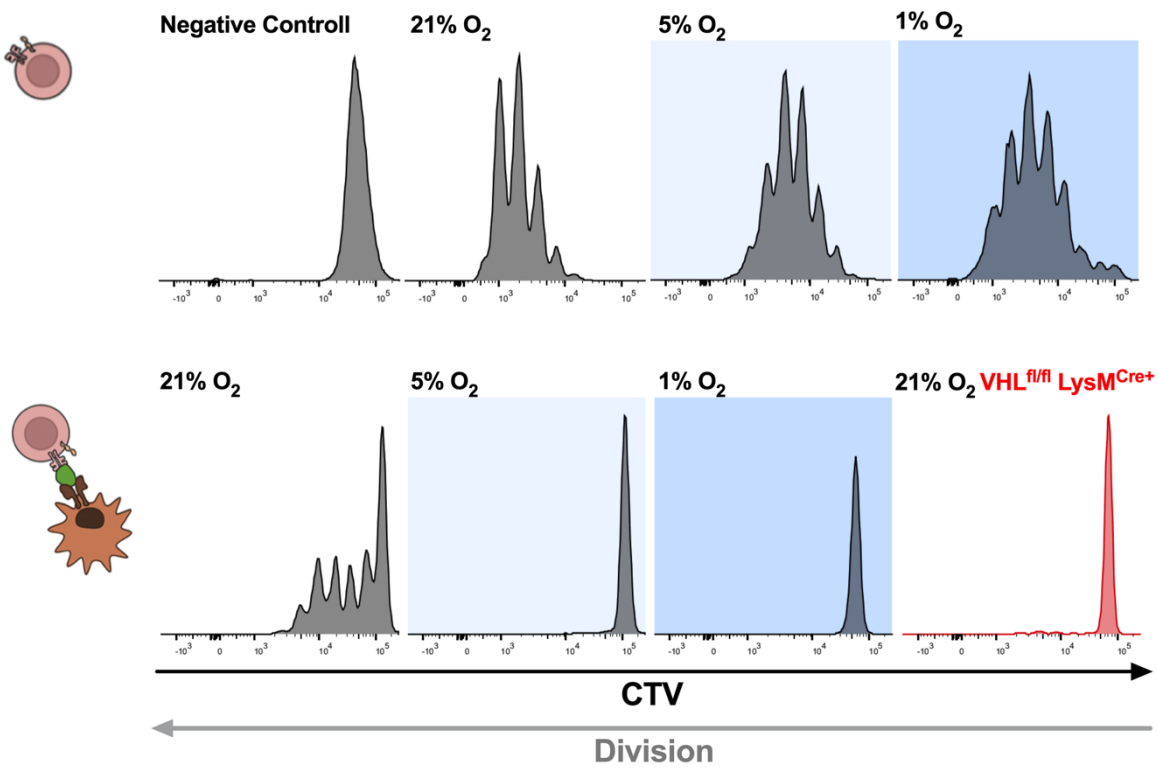

Figure 13: Myeloid cell hypoxia blocks antigen induced T-cell proliferation. Representative figures of CellTrace™ Violet Cell Proliferation stain (CTV), where each peak occurring on the left side represents a cell division comparing single cultures of T cells (top row) or cocultured with antigen presenting BMDMs (bottom row).

In order to understand the mechanism through which myeloid hypoxia is blocking OT-I division and proliferation, a series of antigen-presenting assays were performed with BMDMs lacking HIF-1α, HIF-2α, Arg-1, Arg-2 or NOS2.

In summary (as illustrated in Figure 14), deletion of myeloid HIF-1α or iNOS rescued OT-I ability to divide and proliferate. Furthermore, removal of myeloid HIF-1α and iNOS resulted in higher expression levels of T-cell activation markers, such as Granzyme B and CD44, suggesting that myeloid- NO blocks T-cell activation. To confirm this, T cells were cultured with different doses of a NO donor, diethylenetriamine (NOC-18). Both division and proliferation of activated T cells were blocked by NOC-18 in a dose dependent matter (Figure 4, Paper II).

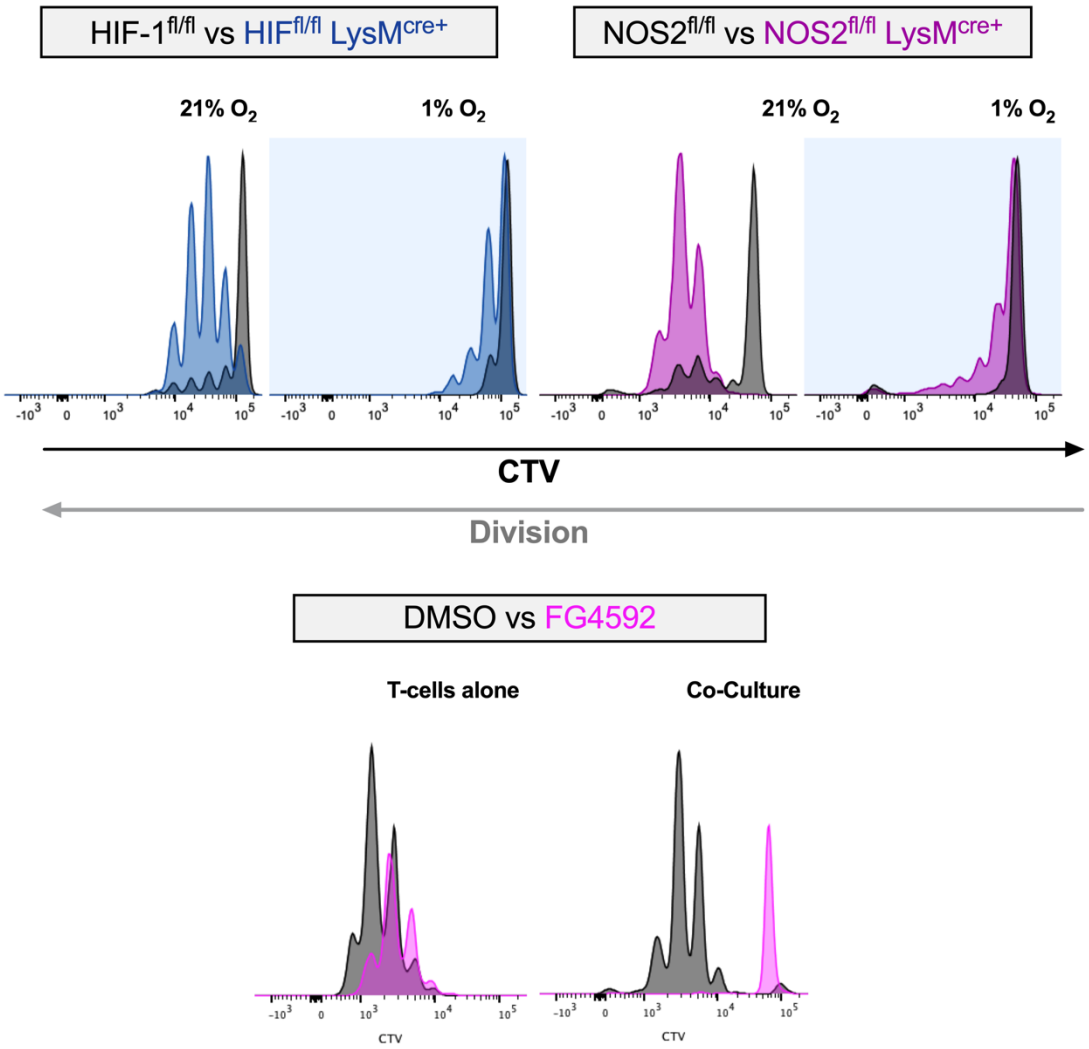

Figure 14: Myeloid cell HIF-1α driven production of NO blocks antigen induced T-cell proliferation. Representative figures of CellTrace™ Violet Cell Proliferation stain (CTV) comparing T-cell proliferation induced by wild type or knockout BMDMs in 21% vs 1% oxygen. Bottom figure compares BMDM treated with vehicle vs 12,5 µM FG4592 in 21% oxygen.

In order to investigate the significance of myeloid cell NO *in vivo,* $NOS2^{fl/fl}LysM^{Cre}$ mice were injected in the tail vein with a melanoma cell line expressing OVA (B16-F10-OVA). By using a metastatic model of melanoma, we were able to observe both the ability of cancer establishment and growth in lungs. Mice lacking myeloid NOS2 had fewer metastatic foci with no difference in size.

Other studies have demonstrated that myeloid cell blockade of already activated T-cells is NO- (Doedens et al. 2010; Bingisser et al. 1998) and cell contact-dependent (Rößner et al. 2005). However, our study confirms the link between myeloid cell hypoxia, NO and the outcome of colonization in the lung. Furthermore, our data suggest that the same cells presenting the antigen also are able to directly inhibit T-cell proliferation, which is interesting in the context of vaccination. Targeting tumors through vaccination against neoantigens is a potential strategy for future treatments (Peng et al. 2019). Furthermore, performing the antigen-presenting assay with a PHD inhibitor replicated the outcome seen in hypoxia (Figure 14).

Our data suggest that myeloid hypoxia and NO could potentially influence vaccine efficiency. Future studies could consist of vaccines combined with molecules inhibiting NO production in order to investigate if decreased amounts of NO would lead to higher efficacy of cancer vaccines. Another aspect for future studies would be the administration routes of vaccines. Perhaps, by administering vaccines in highly oxygenated tissues, such as the lungs, one could obtain stronger T-cell response and consequently receive a more efficient vaccine.

5.3 PAPER III: GLYCOLYTIC RESPONSE TO INFLAMMATION OVER TIME

HIF-1α plays an important role in sepsis and the pathological outcome of sepsis (Vanderhaeghen et al. 2020). Blood pressure changes and hypoglycemia are strongly related to high mortality rates in sepsis (Rattarasarn 1997; Pandey et al. 2014). Previous studies in humans and different murine models of sepsis revealed that absence of HIF-1α signaling in leukocytes correlates with lower mortality rates (Davenport et al. 2016; Peyssonnaux et al. 2007; Yeh et al. 2011). Current literature provides evidence that HIF-1α signaling in leukocytes and myeloid cells has an important role in pathological events during sepsis (Devraj et al. 2017). However, little is known about the underlying mechanism and physiological impact of myeloid HIF-1α. Because of this, a time dependent study in a murine model of sepsis was performed in order to assess the role of myeloid HIF-1α in hypoglycemia and cardiovascular changes in sepsis.

We showed that deletion of myeloid HIF-1α leads to a delayed hypoglycemia during early timepoints. MicroPet imaging with fluorodeoxyglucose (^{18}F-FDG) revealed that most of the reduction in glycolysis was in the heart of HIF-1α$^{fl/fl}$ LysM^{Cre} mice (Figure 15). Likewise, metabolic changes due to deletion of myeloid HIF-1α were observed systemically, HIF-1α$^{fl/fl}$ LysM^{Cre} exhibited a minor reduction in O_2 consumption and carbon dioxide production during the early time points post LPS injection.

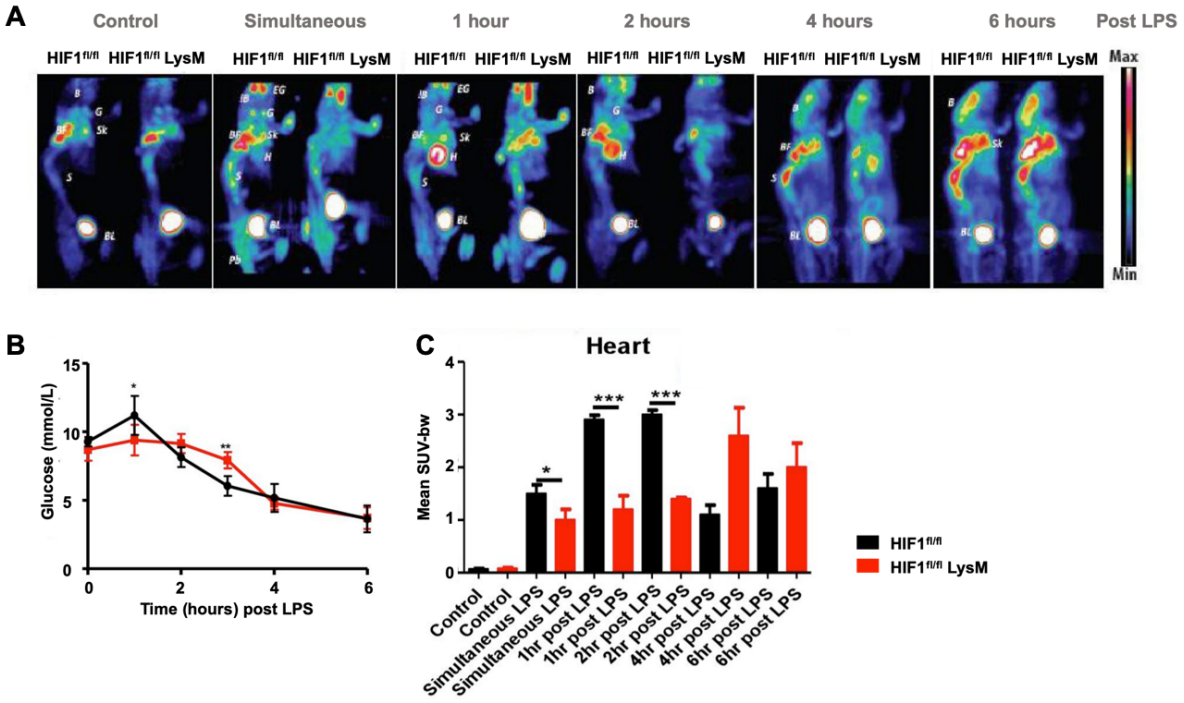

Figure 15: Deletion of myeloid HIF-1α delays LPS induced hypoglycemia. (A) Micro PET imaging glycolytic activity in whole mouse body over time. (B) Blood glucose levels in mice post LPS injection. (C) quantification of glycolytic activity measured with micro pet. Data represented as images, xy graphs or bar graphs. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Statistical analysis was performed with two-way ANOVA and student T test, n = 7–8 mice per group.

As the cytokine storm in sepsis is an essential part of its pathogenesis (Chaudhry et al. 2013); blood was collected at different timepoints to study changes in several cytokines. Absence of myeloid HIF-1α resulted in very low production of IL-6 and IL-5, while production of IL-2 had a lower peak in HIF-1α$^{fl/fl}$ LysM^{Cre} compared to wild type littermates.

Immunological characterization of the cardiac tissue revealed lower frequency of CD4 T cells and an increased frequency of MP in HIF-1α$^{fl/fl}$ LysM^{Cre} mice. Cardiovascular parameters measured by radio telemetry demonstrated a reduction in body core temperature, heart rate and diastolic blood pressure in mice lacking myeloid HIF-1α.

Taken together, the acquired data demonstrate that myeloid HIF-1α is important during early events of LPS-induced sepsis. As demonstrated in Figure 16, myeloid HIF-1α is causing a time-dependent delay in several clinical symptoms associated with sepsis. Another perspective in this study is the dynamics of cardiac glycolysis. The initial uptake of ^{18}F-FDG in HIF-1α$^{fl/fl}$ LysM^{Cre} mice was abolished, while the amount of CD4 T cells was decreased in the cardiac tissue. Beside the increased levels of macrophages, no other changes in immunological populations were observed.

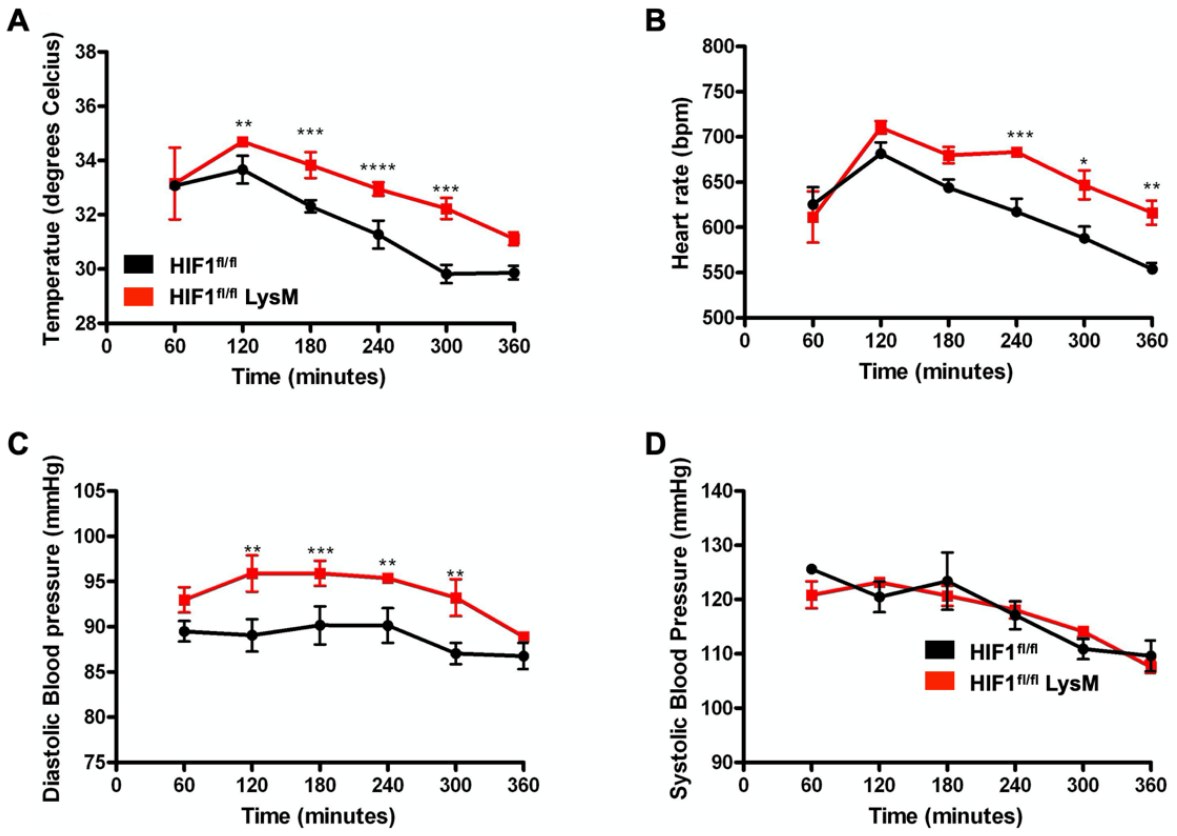

Figure 16: Mice lacking myeloid HIF-1α have dampened development of clinical symptoms associated with LPS induced sepsis. (A) Body temperature, (B) heart rate, (C) diastolic and (D) systolic blood pressure. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Statistical analysis was performed with two-way ANOVA and student T test, n = 4 mice per group.

5.4 COMPLEMENTARY DATA

This section contains data that are currently not a part of any published paper or manuscript. However, results from this section are complementary to this thesis.

Previous studies have shown that removal of MP HIF-1α and HIF-2α resulted in delayed cancer development and tumor growth (Doedens et al. 2010; Susen et al. 2019). Therefore, we hypothesized that increased presence of HIF-1α and HIF-2α would lead to the opposite effects. To validate our hypothesis, we used $VHL^{fl/fl}$ $LysM^{Cre}$ and $FIH^{fl/fl}$ $LysM^{Cre}$ mice; where MP are unable to degrade or regulate the transcriptional function of HIFs in the presence of oxygen, respectively. These experiments were done using a spontaneous breast cancer model MMTV-PyMT crossed with $VHL^{fl/fl}$ $LysM^{Cre}$ and a syngeneic cancer model placed on $FIH^{fl/fl}$ $LysM^{Cre}$ mice. In the spontaneous breast cancer model, mice lacking VHL in the myeloid compartment did not show any difference in tumor onset, survival (Figure 17A) or tumor growth (Figure 17B). To assess the metastatic capacity of these tumors, lungs from tumor-bearing mice were fixed, sectioned and stained with hematoxylin and eosin as previously described (Branco-Price et al. 2012). The number of metastasis or the total metastatic burden (sum of total metastasis per mouse) was not altered by genetic deletion of VHL in myeloid cells (Figure 17C).

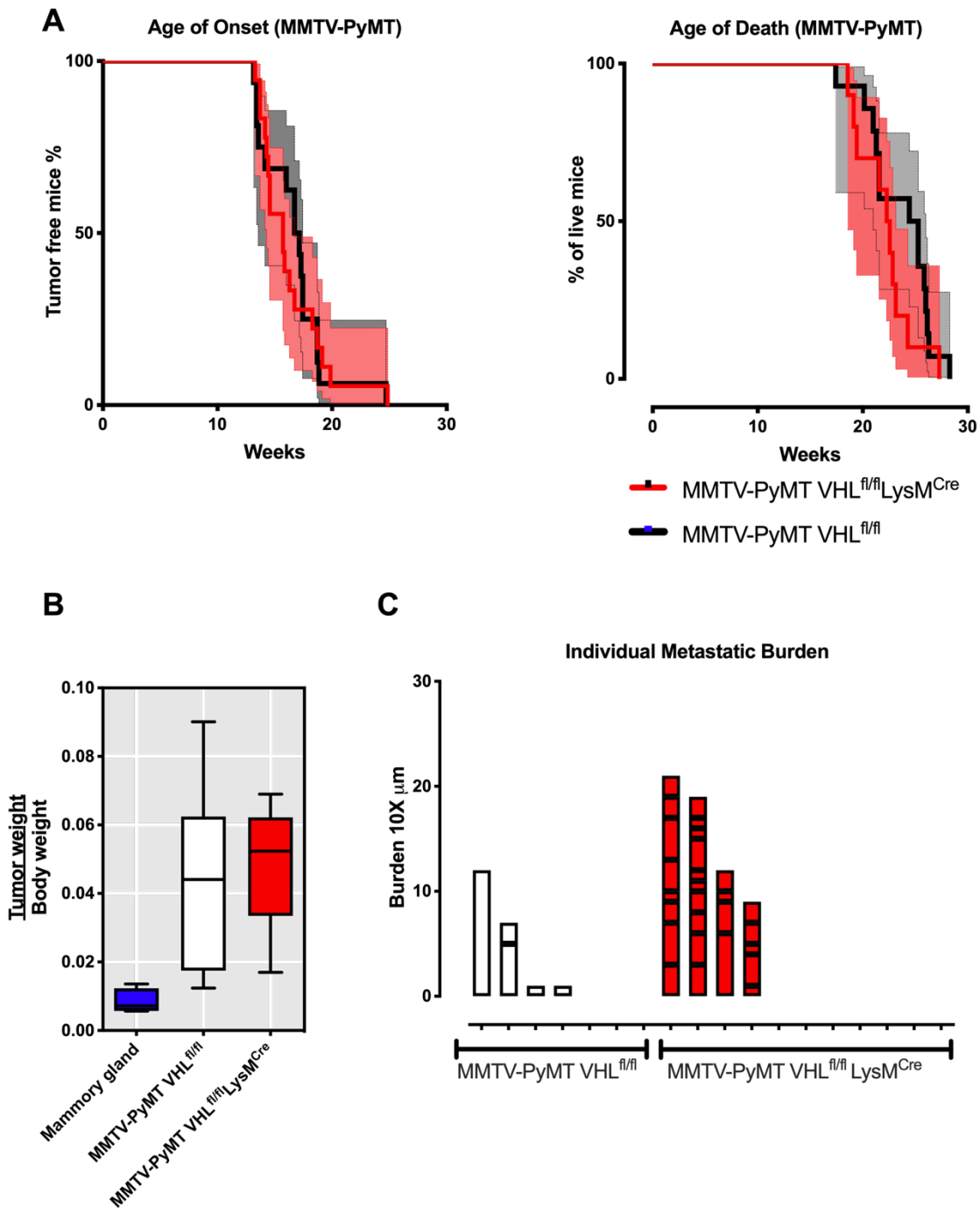

Figure 17: Myeloid deletion of VHL does not affect cancer progression or metastasis in spontaneous breast cancer model MMTV-PyMT. (A) Onset and survival curves of MMTV-PyMT VHL$^{fl/fl}$ and MMTV-PyMT VHL$^{fl/fl}$ LysM^{Cre} mice. Date of sacrifice was determined when total tumor volume reached 1000 m^3. Age of onset, determined when by the day when first palpable tumor was detected (n=16–18). (B) Normalized tumor weight to body weight at week 20 of age (n=4–10). (B) Metastatic number and burden of MMTV-PyMT VHL$^{fl/fl}$ and MMTV-PyMT VHL$^{fl/fl}$ LysM^{Cre} mice. Black lines within bars represent individual metastasis and space between them represents size in µm, each bar is an individual mouse and total height of the bar represents metastatic burden (n=7–11).

FIH$^{fl/fl}$ LysM^{Cre}, where MP HIF-1α and HIF-2α transcriptional activity is not suppressed by oxygen were injected subcutaneously with LLC^{GFP} to assess tumor growth or intravenously in order to assess the metastatic spread. Neither tumor growth (Figure 18A) or pulmonary metastatic establishment (Figure 18B) were different between WT and KO animals. These experiments demonstrated that removal of FIH in myeloid cells does not influence tumor growth or metastasis.

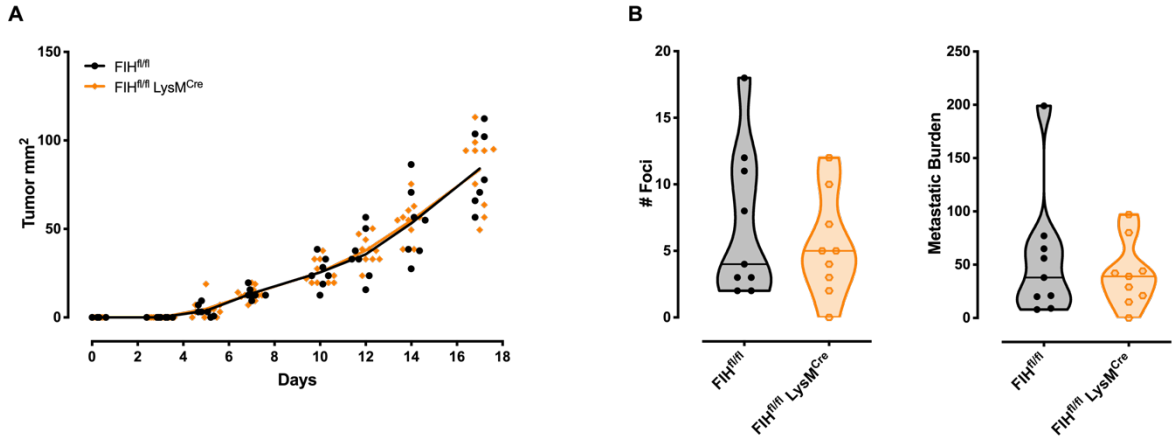

Figure 18: Myeloid deletion of FIH does not affect tumor progression or metastasis in mice injected with LLC^{GFP}. (A) Tumor growth presented as area of tumors in mice injected with 0.5x10^6 LLC^{GFP} cells subcutaneously (n=7–10). (B) Number of foci and metastatic burden of mice injected with 0.2x10^6 LLC^{GFP} intravenously and sacrificed 3 weeks post injection. Metastatic burden is sum of size for all the foci/lung (um) (n=9).

TME changes with different stages of cancer (Hui & Chen 2015), including the frequency and type of tumor-infiltrating lymphoid and myeloid cells (Kather et al. 2018). Because of this, we evaluated time dependent changes of myeloid and lymphoid cells in a syngeneic metastatic model. Lungs and spleens were collected at different time points after intravenous injection of LLC^{GFP}, processed and analyzed by flow cytometry. Our analysis revealed time-dependent changes in several pulmonary populations after injections of LLC^{GFP} (Figure 19). In the myeloid compartment, alveolar macrophages and neutrophils increased in frequency two respectively 21 days post injection of LLC^{GFP}, while monocytes demonstrated a decrease seven days after LLC injection. B cells were the only lymphocyte population to show a significant change in the lungs, with a decrease two days after the injection. However, in spleens, B cells were increased at 21 days, whereas CD4 and CD8 T cells increased in frequency two days after injection and returned to basal levels at the latter timepoints. Compositional shifts of myeloid and lymphoid cells in metastasis at different time points reveals which cell types are important at different stages of metastatic development.

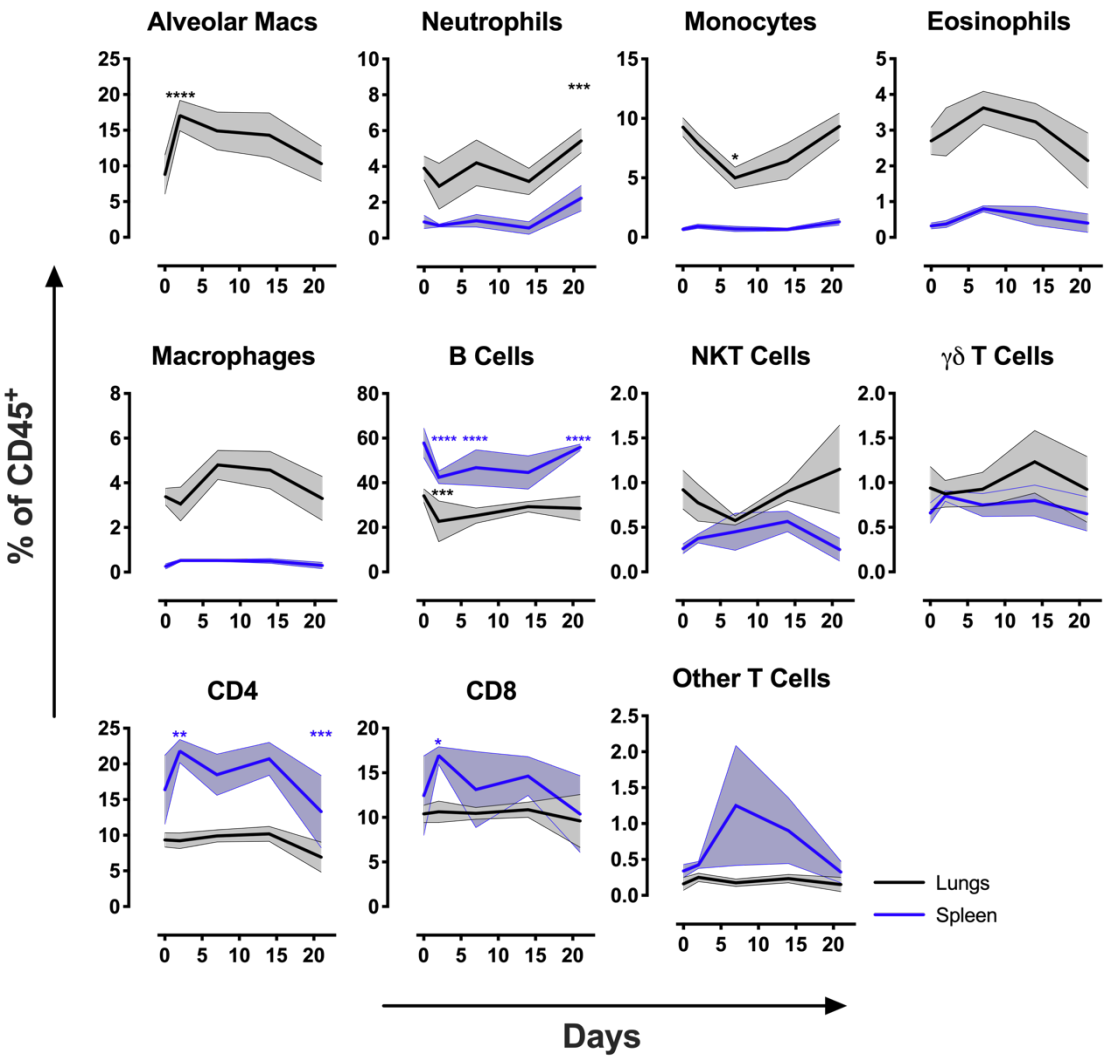

Figure 19: Dynamics of pulmonary populations within the lung post intravenous injections of GFP^{LLC}. Frequencies of myeloid or lymphoid cells normalized to total number of leukocytes. Mice were injected with $0.2x10^6$ LLC^{GFP} intravenously. Perfused lungs and spleens were collected at day 2, 7, 14 and 21. (n=3–5). Data presented as xy graphs with standard deviation, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Statistical analysis was performed with two-way ANOVA and Tukey multiple comparison.

In Paper I, we observed that removal of myeloid VHL leads to increased pulmonary permeability and edema. In an attempt to pinpoint the exact cell type responsible for observed phenotypes in $VHL^{fl/fl}$ $LysM^{Cre}$ mice, we performed a depletion experiment. By injecting tdTomato $LysM^{Cre}$ reporter mice with clodronate liposomes intraperitoneally, pulmonary $CD11b^+$ $CD11c^+$-MP (classically described as macrophages) subpopulation were successfully depleted. However, treatment with clodronate liposomes also resulted in an increased proportion of tdTomato-positive (LysMCre-positive) cells, where neutrophils and monocytes were predominant cell types (Figure 20A). Thereafter, similar clodronate treatment was performed on $VHL^{fl/fl}$ $LysM^{Cre}$, followed by a permeability assay. Depletion of pulmonary macrophages in $VHL^{fl/fl}$ $LysM^{Cre}$ mice resulted in further increased pulmonary permeability (Figure 20B), with no differences observed between wild type littermates treated with liposomes carrying PBS or clodronate. The elevated permeability in clodronate treated $VHL^{fl/fl}$

LysM^{Cre} mice is likely to be caused by increased infiltration of neutrophils and monocytes, compensating for the absence of macrophages. The newly recruited myeloid cells will also be VHL-deficient and therefore enhance pulmonary permeability through HIF signaling. Indirectly, the data in these experiments reinforces the link between myeloid cell hypoxia signaling and pulmonary permeability.

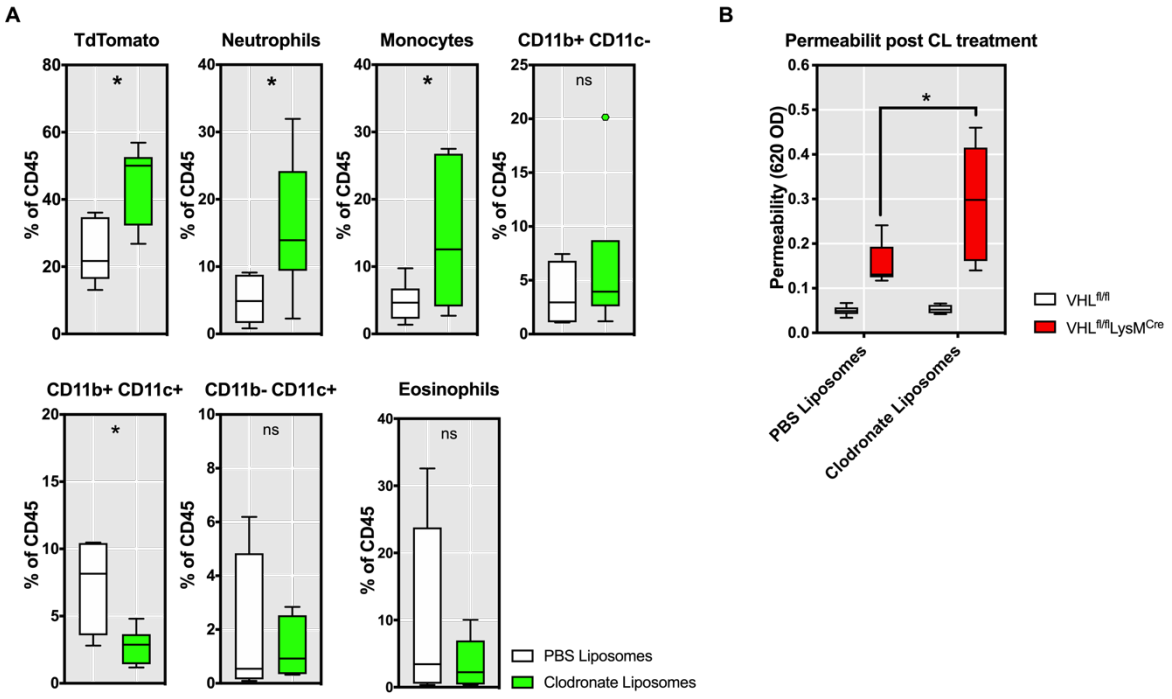

Figure 20: Depletion of macrophages by clodronate liposomes increases frequency of other myeloid cells in the pulmonary tissues and enhances permeability induced by myeloid cells lacking VHL. (A) Frequencies of LysM active cells and myeloid cells normalized to total number of leukocytes. Mice were injected with liposomes either containing PBS or clodronate intra-peritoneally at day 0 and 3, lungs were perfused and collected at day 4. Dosage was 0,2 mL per mouse (n=6–7). (B) Pulmonary permeability of mice treated with liposomes containing PBS or Clodronate (n=4–6). *= $p < 0.05$, ns = not significant. Statistical analysis was performed with unpaired T test.

HIF-1α and HIF-2α contribute to different functions of BMDM; the pro-inflammatory functions are driven by HIF-1α, while the anti-inflammatory functions by HIF-2α (Takeda et al. 2010). In order to understand the impact of deregulated HIF signaling in BMDM, RNA-sequencing was performed on BMDM derived from VHL$^{fl/fl}$ and VHL LysM^{Cre} mice. This was done with BMDM in three different conditions: untreated, stimulated with pro-inflammatory cytokines (TNFα and IFNγ) and anti-inflammatory cytokines (IL4 and IL13). Comparing different conditions, principal component analysis revealed that cytokine treatment had a stronger impact on BMDM transcriptome than deletion of VHL (Figure 21A). Although cytokine treatments had the strongest impact on BMDM transcriptome, our aim was to understand the specific role of HIF signaling in each condition. Therefore, comparison of expressional profiles were performed between VHL$^{fl/fl}$ with VHL LysM^{Cre} BMDM within each treatment (Figure 21B), followed by comparison significant hits between the different treatments (Figure 21C).

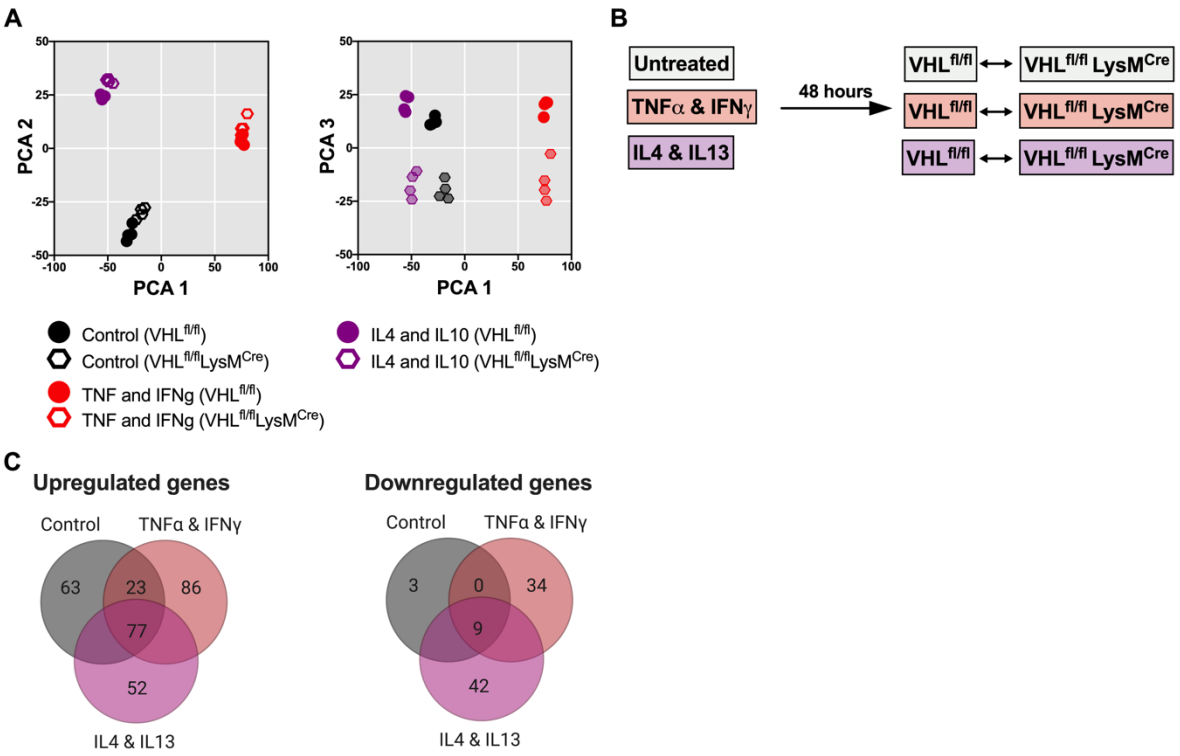

Figure 21: Deletion of VHL in bone marrow derived myeloid cells results in differential gene expression in different conditions. RNA sequencing performed on bone marrow myeloid cells with different treatments. (A) Top three principal component analysis separating samples primarily by treatment, secondly by deletion of VHL. (B) Illustration of different conditions used for RNA sequencing. Differential gene expression analysis was performed comparing BMDMs from $VHL^{fl/fl}$ with $VHL^{fl/fl}$ $LysM^{Cre}$ mice. (C) Venn diagram illustrating number of common and unique genes differentially expressed between different conditions. Cut off was done at 2-fold difference and FDR 0.01. n=4

The largest proportion of genes that were upregulated due to deletion of VHL were shared between all conditions. These were genes involved in cellular processes of O_2 sensing, hypoxia and metabolism (Figure 22). Nonetheless, deletion of VHL resulted in condition-specific transcription profiles. In untreated BMDM, loss of VHL resulted in upregulation of genes involved in inflammatory and immunological processes (Figure 23). In contrast, combination of VHL deletion and treatment with TNFα and IFNγ resulted in downregulation of genes involved in phosphorus process (kinase activity and protein phosphorylation) and upregulation of genes involved in negative regulation of cell proliferation and stress response (Figure 24). Furthermore, combination of VHL deletion and IL4 and IL13 treatment resulted in transcriptional changes of genes involved in cellular adhesion (Figure 25). The preliminary transcriptional profiling analysis on VHL knockout BMDM in different conditions shown here reveals that the transcriptional response to hypoxia in myeloid cells varies according to the stimulus applied.

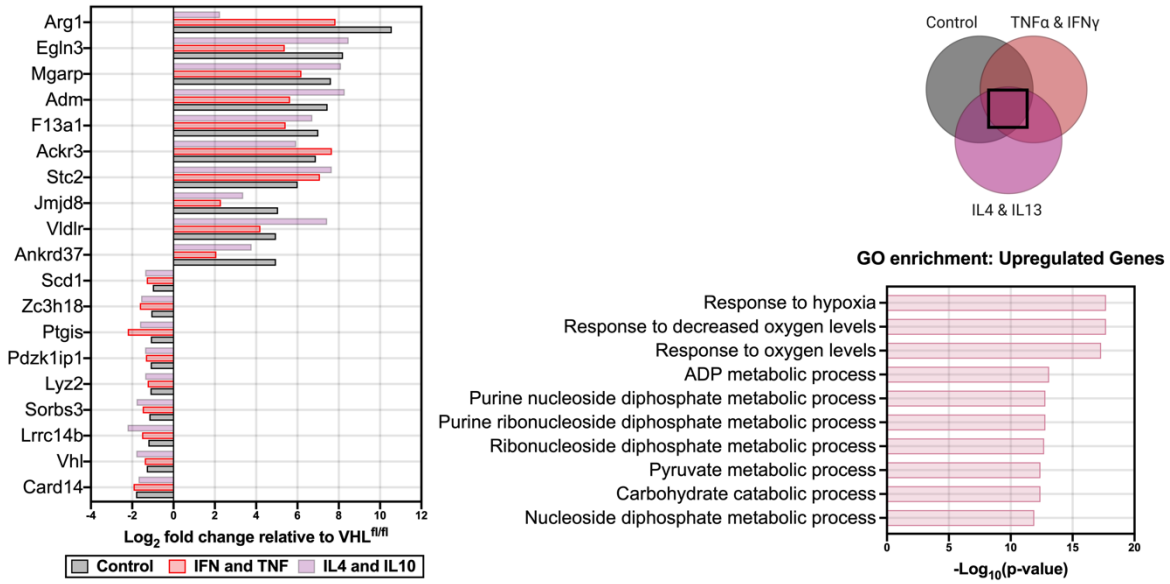

Figure 22: Genes of hypoxia signaling are deregulated by VHL deletion in myeloid cells independent of culturing conditions. Log_2 fold change of most deregulated genes by VHL deletion and the most significant GO-terms.

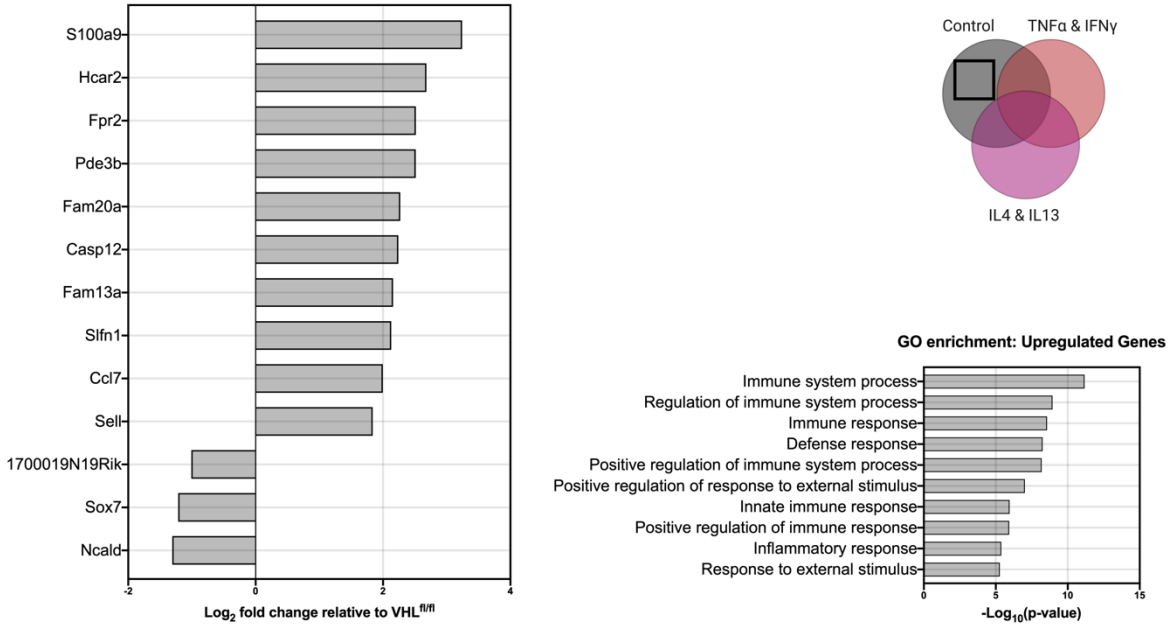

Figure 23: Loss of VHL enhances expression of genes involved in immune response in untreated BMDMs. Log_2 fold change of most deregulated genes by VHL deletion and the most significant GO-terms.

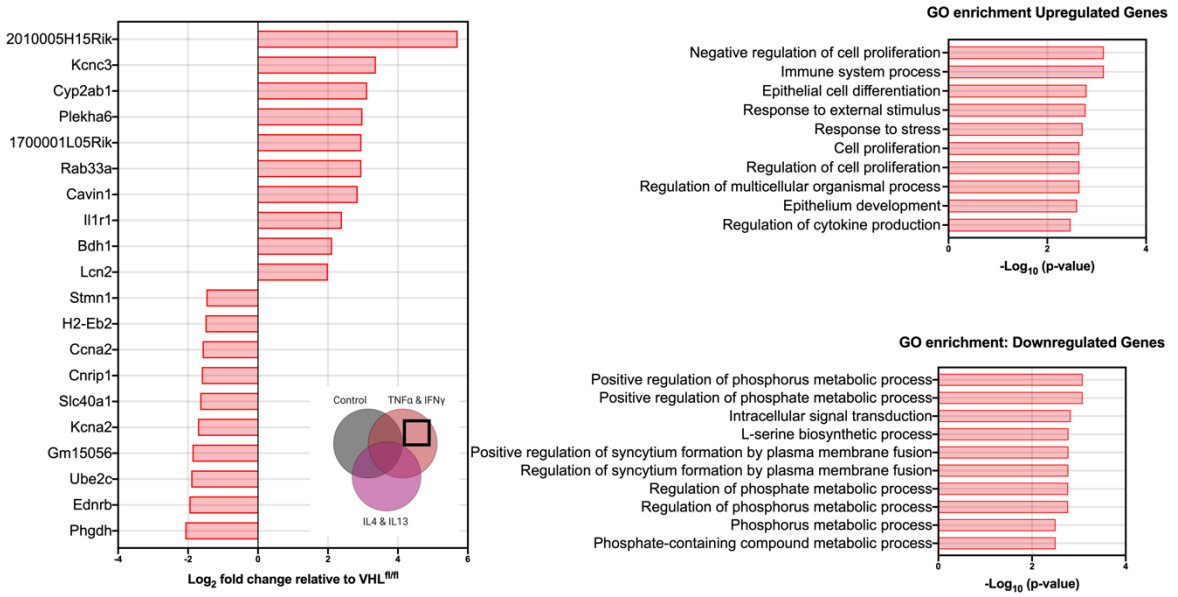

Figure 24: Loss of VHL in TNFα and IFNγ treated BMDMs preliminary deregulates genes involved in metabolic processes and cellular proliferation. Log_2 fold change of most deregulated genes by VHL deletion and the most significant GO-terms analysis of downregulated and upregulated genes.

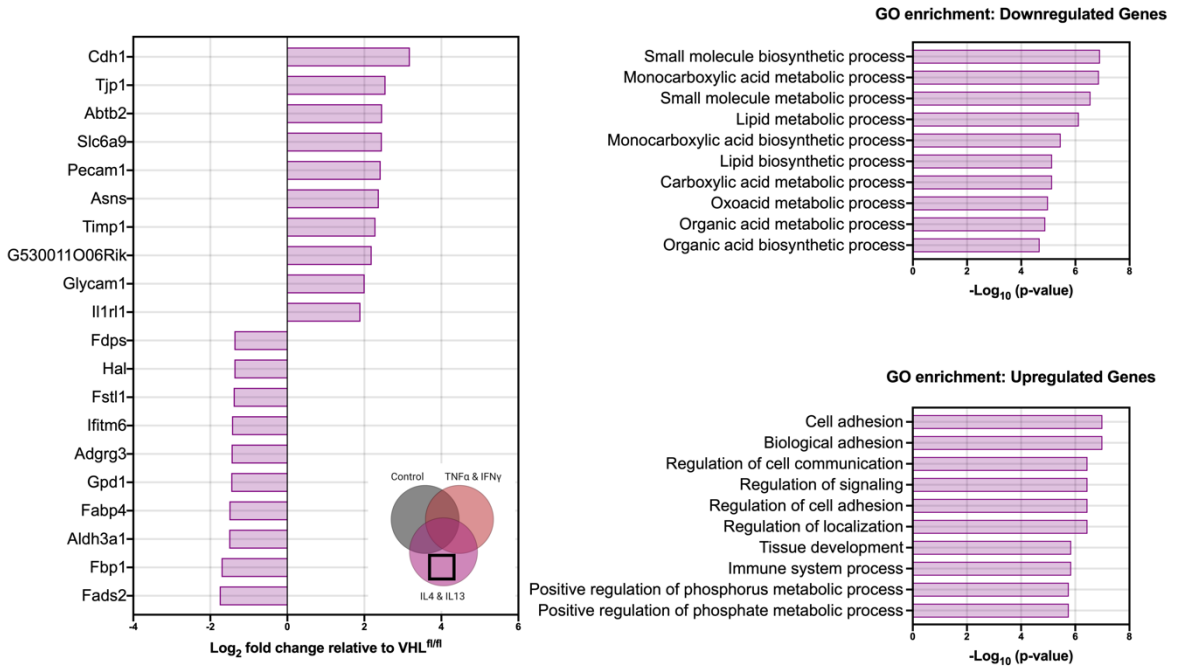

Figure 25: VHL deletion in BMDMs treated with IL4 and IL13 results in altered metabolism and cell adhesion. Log_2 fold change of most deregulated genes by VHL deletion and the most significant hits of GO enrichment analysis of downregulated and upregulated genes.

6 SUMMARY AND CONCLUSIONS

Oxygen sensing in myeloid cells seems to be involved in several pathological conditions driven by inflammation (Palazon et al. 2014). Hypoxia and inflammation are tightly linked as most inflammatory conditions lead to challenged O_2 delivery and increased O_2 demand as recruited inflammatory cells increase the cellular density in the tissue (Schwartz et al. 2011). The inflammatory signature observed in individuals suffering from HAPE (Mishra et al. 2015) are an example where hypoxia itself is enough to trigger an inflammatory response. Although data gathered from HAPE patients does not provide enough evidence to definitely link myeloid inflammation to the pathological progress of HAPE, animal models and experimental conditions do demonstrate a strong link. The clearest examples of the link between inflammation and environmental hypoxia are observed in studies where removal of MP or MP HIF-1α in mice weakens the progression of HAPE (Kojima et al. 2019; Vergadi et al. 2011).

Our data contributes to the evidence that links inflammation and HAPE. Removal of VHL in myeloid cells leads to development of HAPE-like symptoms. Collectively, data gathered in our study demonstrates HAPE-like signature spanning from gene expression to physiological outcomes, therefore we propose that $VHL^{fl/fl}$ $LysM^{Cre}$ mice could serve as a model of HAPE.

In cancer, the literature presents a complex role of myeloid HIF-1α and HIF-2α. The heterogeneity of cancer itself and the discrepancy of murine models contribute to the complexity of current knowledge. Overall, a majority of studies om MP-HIF-1α signaling in cancer suggest that both MP HIF-1α and HIF-2α contribute to the progression of cancer; MP-HIF-1α seems to be important for spontaneous development of tumors (Henke et al. 2016; Takikawa et al. 2019), whereas MP HIF-2α may assist in the pathogenesis of already established tumors (Imtiyaz et al. 2010; Susen et al. 2019). Thus, MP-HIF-1α drives the early establishment of tumors, while MP HIF-2α drives the later stages of cancer progression. Data from paper II and the complementary data included in this thesis supports MP-HIF-1α role in early establishment of cancer. MPs are capable to aid the adaptive immune system in clearance of tumors by antigen presentation. As observed in paper II, MP hypoxia and HIF-1α signaling blocks activation of T cells through NO. Furthermore, this process seems to be important in early establishment of tumor growth as removal of MP-NOS2 affects the initial establishment and not the growth of already established tumors. Therapeutically, removal of HIF-1α/NOS2 signaling could potentiate the efficacy of vaccination against tumors and other diseases. However, drugs that activate HIF signaling might contribute to the establishment of new tumors by blocking the activation of the adaptive immune system.

Complementary data demonstrates some controversy about the role of MP HIF signaling in cancer progression, as removal of HIF regulation does not demonstrate any effect. However, as a combination of cancer inflammation and hypoxia drive HIF signaling in MPs (McKeown 2014; D'Ignazio et al. 2017), removal of FIH or VHL might not contribute to further increase of MP HIF signaling. Additionally, the cancer models used in our studies are known to be aggressive, which could potentially hide the effects of insufficient activation of the adaptive immune system by MPs.

Sepsis is another condition where a blockade of HIF signaling could serve as a possible therapeutic target. Earlier studies in mice have demonstrated that removal of HIF signaling decreases the severity of sepsis and increases the survival (Peyssonnaux et al. 2007), while in humans, groups with high mortality rates in sepsis have enhanced HIF expression (Davenport et al. 2016). Observations in Paper III suggest that targeting MP HIF-1α could potentially decrease organ damage caused by hypoglycemia and hypotension in septic shock. These observations also confirm that MP hypoxia signaling has consequences on the physiological level.

HAPE, cancer and sepsis are three substantially different pathological events with their origins in environmental hypoxia, genetic mutations and infection. Although triggered by different causes, a common denominator of all three diseases is that myeloid cells in hypoxic environments are present, play a role and can even be the drivers of the disease progression. Shared signaling pathways, cytokines and physiological outcomes between these diseases originate from MPs and hypoxia. In this aspect, MPs and hypoxia are serving as a junction point for progression of HAPE, cancer and sepsis (Figure 26)

As most junction points are complex, the role of HIF signaling in MP function is no exception. Transcriptional data in figures 22-26 show that the outcome of MP hypoxia is stimulus dependent; expressions of more than 50 genes are condition-specific, even though they originate from the same genetic manipulation. Together with the plastic nature of MPs, more detailed studies of MP hypoxia are needed to truly understand the full potential of HIF signaling in myeloid cells and consequences for the physiological and pathological outcomes.

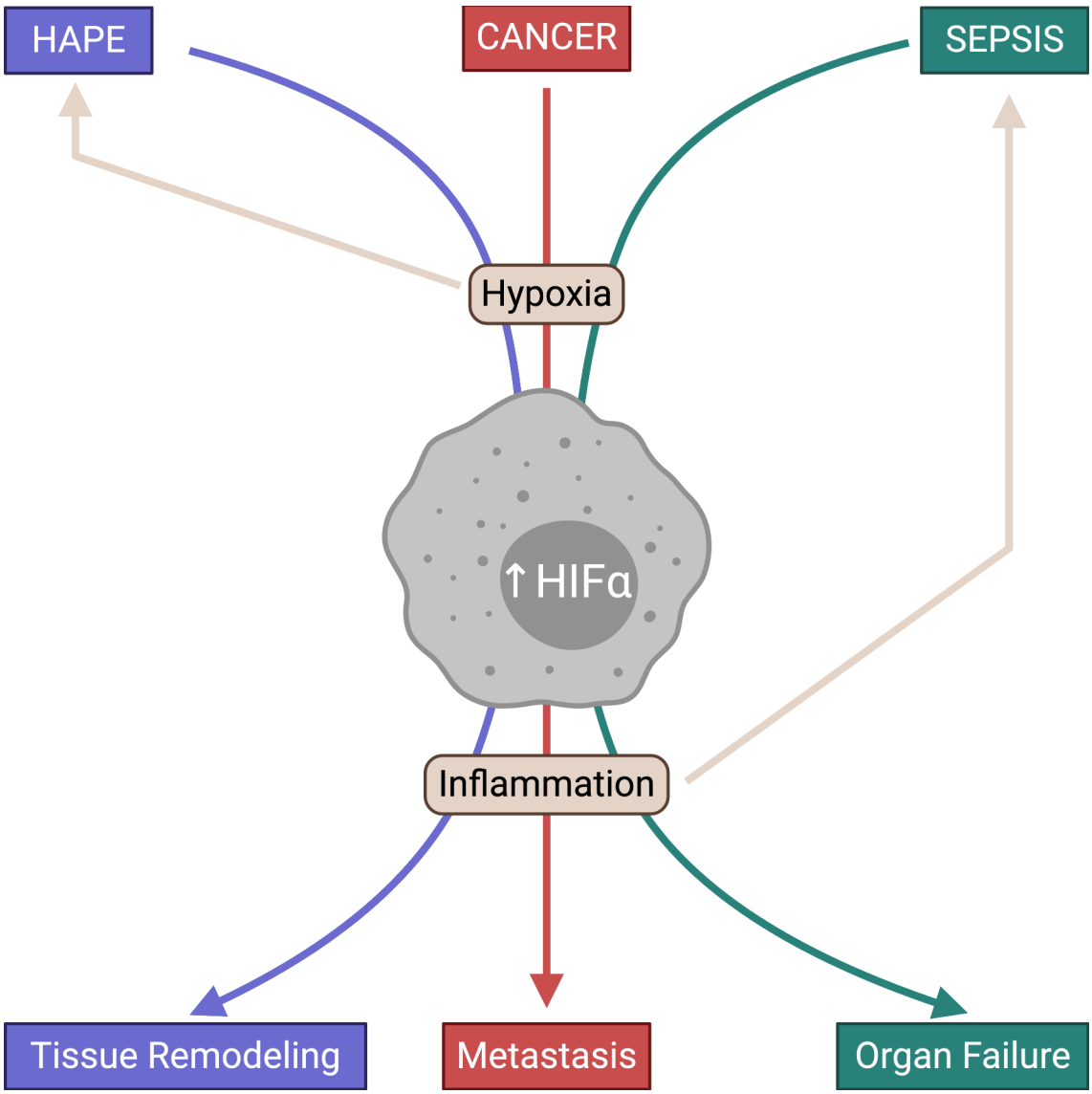

Figure 26: HAPE, cancer and sepsis are very different diseases with MP hypoxia as a junction point.

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Afterword: This thesis was written during the COVID-19 pandemic caused by SARS-CoV-2. A time point marked by reporting of daily deaths and confirmed cases, social distancing, travel restrictions, lockdowns and economical disturbances. We are currently living in interesting times, where the outcome of the pandemic is still to be seen.

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