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STUDIES ON THE ROLE OF HIF-1 IN THE CONTROL OF INFECTION WITH *MYCOBACTERIUM TUBERCULOSIS*

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STUDIES ON THE ROLE OF HIF-1 IN THE CONTROL OF INFECTION WITH *MYCOBACTERIUM TUBERCULOSIS*

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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POPULAR SCIENCE SUMMARY OF THE THESIS

Tuberculosis (TB) has tormented humans for thousands of years as can be seen in mummies with TB, and is still a global public health problem, especially in developing countries. TB is caused by *Mycobacterium tuberculosis (M. tuberculosis),* an intracellular bacterium. In 2021, 10.6 million people developed TB and 1.6 million people died from it. A healthy individual is infected if he inhales bacteria transmitted through aerosols from a TB patient through coughing, sneezing or shouting. While many individuals may clear the bacteria after infection, 5% of the productively infected individuals develop primary TB soon after infection, while 95% of individuals develop latent TB infection in which with no symptoms of disease but at risk of reactivation which occurs in ca 5% of them. To prevent from TB, babies are vaccinated with BCG, the only licensed vaccine against TB, after birth. Unfortunately, BCG is not able to wholly provide protection against TB, particularly in adults. Why individuals have such diverse risks of developing TB is not completely understood. People living with human immunodeficiency virus or diabetes mellitus (DM) have increased risk of infection with TB strongly suggesting that the immune system-bacterial interactions are major players in the outcome of the infection. An improved understanding of immune factors controlling TB and understanding mechanisms behind comorbidity with DM will help in the design of better tools to control this infection.

We have a powerful immune system that protects us from all kinds of invaders, for example, viruses, bacteria and worms. In our body, two major types of immune cells, which are T cells and macrophages, are essential for clearance of *M. tuberculosis* in the lung. Specifically, macrophages engulf *M. tuberculosis* during infection and send signals to T cells which then secrete molecules activating macrophages to kill bacteria through their antimicrobial activities.

Transcription factors control the expression of genes in eukaryotic cells so they can adapt to different environments and stresses. Hypoxia inducible factors (HIF) are a family of transcription factors that control the response to cells in a low oxygen environment. HIF can be involved in regulation of the responses of immune cells even when oxygen is not low. It has been shown HIF-1 in T cells contributes to the protection against viruses and tumors by others. HIF-1 in macrophages is also involved in control of different intracellular infections including *M. tuberculosis.*

In this thesis, we focus on the role of HIF-1 in modulating T cell and macrophage responses during *M. tuberculosis* infection, BCG immunization and comorbidity with DM. We have used a mouse model which mimics the infection in man.

In **paper I**, we studied the infection outcome of genetically modified mice in which HIF-1 was deficient or stabilized via deletion of HIF-1 and VHL respectively in T cells. The aim of the study was to evaluate the role of HIF-1 in T cells during *M. tuberculosis* infection and BCG vaccination. We did not find any effects of HIF-1 deficiency on T cell responses or outcome of infection. Instead, HIF-1 stabilization diminished differentiation, proliferation and survival

by affecting T cell activation. T cell function is fundamental for control of *M. tuberculosis* infection. The excess of HIF-1 (which can occur for example in hypoxia) will impair such protection. The mice with HIF-1 stabilization in T cells died eventually because poorly functional T cells response cannot control the infection. T cell responses in these mice did not properly respond to BCG vaccination either.

In general, BCG is administered via intradermal rout and then memory T cells are expected to accumulate in the lung and provide protection when the vaccinated individual is infected with *M. tuberculosis*. Recently, mucosal vaccination of BCG in mice was shown to provide a superior control against infection compared to intradermal vaccination. Among diverse T cell populations, resident memory T cells (T_{RM}) in the lung may be especially important for conferring protection. In **paper II**, we investigated how T_{RM} cells were generated and maintained during infection following mucosal and intradermal vaccination. We found that antigen-specific T_{RM} accumulated and proliferated in the lung rather than in lymph nodes, which following the immunological dogma are the site of T cell priming and also activation during infection and vaccination. Throughout infection, antigen-specific T cells in the LN were negligible as compared to those in the lungs. Moreover, more antigen-specific T_{RM} cells were generated in lung from mice vaccinated directly in the respiratory airways than when vaccinated subcutaneously.

As mentioned above, HIF-1 contributes to responses of macrophages in infection. However, HIF-1 function was shown to be impeded in DM patients. Thus, we hypothesized that HIF-1 played a role in modulating function of macrophages in TB-DM comorbidity. In **paper III**, we showed reduced HIF-1 regulated immune responses and more bacteria levels in macrophages cultured under high glucose or after a carbonyl stress, hallmarks of DM. However, macrophages in which HIF-1 was chemically stabilized, bacterial control and protective responses were restored. responses and bacteria growth in macrophages. The stabilization of HIF-1 in macrophages can be a target to diminish the risk of TB during comorbidity with DM.

To summarize:

1. HIF-1 stabilization in T cells increases susceptibility to *M. tuberculosis* infection in mice
2. Mycobacteria-specific T_{RM} cells are generated in the lung rather than in lymph nodes during *M. tuberculosis* infection and mucosal BCG vaccination.
3. High glucose condition in DM may influence macrophages´ function through compromising HIF-1

ABSTRACT

Mycobacterium tuberculosis (*M. tuberculosis*), the causative agent of tuberculosis (TB), is responsible for most deaths caused by a bacterium globally. Two main reasons for this are that (*Bacille Calmette–Guérin*) BCG, the only available vaccine against TB, cannot provide a full protection against TB in adults and that antibiotic treatment is hampered by the increase of drug resistances to *M. tuberculosis*. In addition, it is still not completely understood why some people are more prone to developing active TB after infection with *M. tuberculosis*, while others remain asymptomatic. As the co-infection with the human immunodeficiency virus or the comorbidity with diabetes mellitus (DM) increases the risk of TB reactivation, this indicates that interactions between host immune factors and *M. tuberculosis* determine the outcome of infection. The interaction would lead to the development of granulomas composed of macrophages, granulocytes, lymphocytes and fibroblasts. *M. tuberculosis* control is primarily mediated by macrophages, the main host cell of intracellular *M. tuberculosis*, that are activated by mycobacteria-specific CD4 T_H1 cells.

The studies presented in this thesis analyze molecular factors that may control the response of macrophages and T cells to *M. tuberculosis* infection. For increased understanding of generated T cell responses upon vaccination, we studied the phenotype of T cells and their localization after mucosal and distal BCG immunization. Studies were evaluated by using *M. tuberculosis*-infected mice and were further complemented with mechanistical in vitro studies.

Studied molecules in **paper I** are the hypoxia-inducible factors (HIFs), transcription factors regulating the adaptation to hypoxia of different cell populations, which are negatively regulated by von Hippel-Lindau factor (VHL). One of the transcription factors, HIF-1, has been broadly studied in immune cells. It can mediate metabolic and immunoregulatory responses of immune cells. Therefore, we investigated the role of HIF-1 overexpression in CD4 T cells during infection with *M. tuberculosis* and found that mice with VHL deficiency in T cells (*Vhl cKO*) were more susceptible to *M. tuberculosis* infection. In contrast, mice with HIF-1 deficiency in T cells behave similar to wild type control during *M. tuberculosis* infection, which suggests that HIF-1 deficiency plays a redundant role in T cells in *M. tuberculosis*-infected mice. The increased susceptibility of *Vhl cKO* mice is attributed to a less *M. tuberculosis*-specific T cells accumulated in the lungs that show an impaired cell proliferation, a perturbed differentiation and an amplified expression of inhibitory receptors. We also observe blunt T cell responses in *Vhl cKO* mice immunized with BCG. Moreover, VHL plays an essential role in MYC activity, cell activation, growth, expansion and survival of CD4 T cells activation via TCR signaling. The impaired responses of VHL deficient T cell are reversed by HIF-1 deletion, indicating that the increased susceptibility of *M. tuberculosis* infection and impaired T cell responses in *Vhl cKO* mice is attributed to HIF-1 stabilization in T cells.

In **paper II**, we analyzed the localization and differentiation of mycobacteria-specific CD4 and CD8 T cells after subcutaneous (s.c) and intratracheal (i.t.) immunization with BCG and aerosol infection with *M. tuberculosis* in mice. We find mycobacteria-specific and proliferating CD4 and CD8 T cells accumulating in the lung but not in the lung draining lymph node (dLN)

at several time points after infection or immunization. After *M. tuberculosis* infection CD4 and CD8 T cells that secrete IFN-γ after stimulation with different mycobacterial peptides or PPD (a mycobacterial protein extract) are found in lungs, but not in dLN. Resident memory CD4 and CD8 T cells (T_{RM}) expressing PD-1 that are mycobacteria-specific accumulate in the lung parenchyma after aerosol infection and i.t. -rather than s.c.- immunization. Fingolimod treatment inhibiting recirculation indicates that T_{RM} are generated in the lung parenchyma after i.t. BCG booster. Collectively, our data suggests mycobacteria-specific T cells during mucosal BCG immunization and *M. tuberculosis* infection may be generated and/ or expanded in the lung.

DM is positively associated with active TB development, but the mechanisms remain unclear. In **paper III**, we investigated the role of HIF-1 in bone marrow-derived macrophages (BMM) during *M. tuberculosis* or BCG infection. We observe expression of HIF-1, HIF-1 regulated genes involved in metabolic transcripts and immune molecules are significantly induced in BMM and lung from mice with mycobacteria infection. Deferoxamine (DFO) treatment that mimics hypoxia further enhances HIF-1-regulated responses and restricted bacterial growth both in vitro and in vivo. HIF-1-regulated responses are diminished and the intracellular bacterial load in BMM is increased by the treatment with high glucose levels or methylglyoxal (MGO), a highly reactive dicarbonyl metabolite enriched in DM. In accordance with this, more levels of *M. tuberculosis* loads, and lower levels of HIF-1-regulated genes were shown in lungs from hyperglycemic $Lepr^{db/db}$ mice infected with *M. tuberculosis*. The effects of high glucose or MGO on intracellular *M. tuberculosis*-growth and HIF-1-regulated transcripts in BMM were reverted by addition of DFO. Besides, loss of *Hif1a* in BMM diminishes expression of HIF-1-regulated inflammatory and metabolic transcripts after mycobacterial infection under normal or high glucose conditions. In summary, our data suggests that HIF-1 could be a potential target for improved control of *M. tuberculosis* during DM.

In conclusion, we found that:

4. Stabilized HIF-1 in T cells hinders *M. tuberculosis* infection control by impairing T cell activation.
5. Mycobacteria-specific T cells accumulate in the lung but not in the dLN during *M. tuberculosis* infection or mucosal BCG immunization.
6. Although HIF-1 in macrophages plays a protective role against *M. tuberculosis*, its function and levels are reduced under hyperglycemia and carbonyl stress.

LIST OF SCIENTIFIC PAPERS

I. **Liu R**, Muliadi V, Mou W, Li H, Yuan J, Holmberg J, Chambers BJ, Ullah N, Wurth J, Alzrigat M, Schlisio S. HIF-1 stabilization in T cells hampers the control of Mycobacterium tuberculosis infection. Nature communications. 2022 Sep 5;13(1):1-20.

II. Basile JI*, **Liu R***, Mou W, Gao Y, Carow B, Rottenberg ME. Mycobacteria-specific T cells are generated in the lung during mucosal BCG immunization or infection with mycobacterium tuberculosis. Frontiers in immunology. 2020 Oct 22;11:566319. *: Shared first authors

III. Terán G, Li H, Catrina SB, **Liu R**, Brighenti S, Zheng X, Grünler J, Nylén S, Carow B, Rottenberg ME. High Glucose and Carbonyl Stress Impair HIF-1-Regulated Responses and the Control of Mycobacterium tuberculosis in Macrophages. Mbio. 2022 Sep 19:e01086-22.

Scientific papers not included in the thesis

I. Gao Y, **Liu R**, He C, Basile J, Vesterlund M, Wahren-Herlenius M, Espinoza A, Hokka-Zakrisson C, Zadjali F, Yoshimura A, Karlsson M. SOCS3 expression by thymic stromal cells Is required for normal T cell development. Frontiers in immunology. 2021 Mar 18;12:642173.

CONTENTS

LIST OF ABBREVIATIONS

AGEs	Advanced glycation endproducts
APCs	Antigen-presenting cells
ARNT	Hydrocarbon receptor nuclear translocator
BCG	Bacillus Calmette-Guérin
BMM	Bone marrow-derived macrophages
CCL	CC chemokine ligand
CCR	CC chemokine receptor
CTL	Cytotoxic T lymphocytes
CX3CR1	CX3C motif chemokine receptor 1
CXCR	C-X-C chemokine receptor
DCs	Dendritic cells
DFO	Deferoxamine
dLN	Draining lymph node
DM	Diabetes Mellitus
ER	Endoplasmic reticulum
ESAT-6	Early secreted antigenic target of 6 kDa protein
Fe	Iron
GFP	Green fluorescent protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HIF	Hypoxia Inducible Factor
HIV	Human immunodeficiency virus
Hsp	Heat shock protein
iBALT	Inducible bronchus-associated tissues
ICOS	Inducible T cell co-stimulator
IFN- γ	Interferon γ
IL	Interleukin
iNOS	Inducible nitric oxide synthase
ITAMs	Immunoreceptor tyrosine-based activation motifs
i.t.	Intratracheal
LAT	Linker for activation of T cells

LCMV	Lymphocytic choriomeningitis virus
LPS	Lipopolysaccharide
LTBI	Latent TB infection
MAPK	Mitogen-activated protein kinase
MHC	Major histocompatibility complex
M-CSF	Macrophage colony-stimulating factor
MGO	Methylglyoxal
mTOR	Mammalian target of rapamycin
M. tuberculosis	*Mycobacterium tuberculosis*
NO	Nitric oxide
OXPHOS	Oxidative phosphorylation
PD-1	Programmed cell death protein 1
PDK1	3'-phosphoinositide-dependent kinase-1
PHDs	Prolyl hydroxylases
PLC	Phospholipase C
PMA	Phorbol myristate acetate
PRRs	Pattern recognition receptors
PO_2	Pressure of oxygen
RNIs	Reactive nitrogen intermediates
ROIs	Reactive oxygen intermediates
RORγt	Retinoic acid-related orphan receptor gamma t
S-2HG	S-2-hydroxyglutarate
s.c.	Subcutaneous
SLO	Secondary lymphoid organs
SLP	Leukocyte phosphoprotein of 76 kDa
STAT	Signaling transducer and activator of transcription
TB	Tuberculosis
T_{CM}	Central memory T cell
TCR	T-cell receptor
T_{EM}	Effector memory T cells
T_{FH}	T follicular helper cells

TGF-β	Transforming growth factor-β
T_H	T helper cells
Treg	Regular T cells
T_{RM}	Resident memory T cells
VEGF	Vascular endothelial growth factor
VHL	Von-Hippel Lindau tumor suppressor
WT	Wild type

1 INTRODUCTION

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (*M. tuberculosis*), kills about 1.5 million people each year. 90-95% of infected individuals develop a latent TB infection. Comorbidity with diabetes mellitus (DM) can increase the risk of developing active TB by 2-4-fold. The underlying mechanisms of immune responses behind the TB and DM comorbidity are still unclear.

M. tuberculosis bacteria infect mainly macrophages, in which they can proliferate. Since it is intracellular, antibodies do not play a major role in controlling the infection. Instead, T cells, and especially CD4 T cells, participate in protective immune responses during the infection. The infection with mycobacterium including virulent strain *M. tuberculosis* and attenuated strain *Bacillus Calmette-Guérin* (BCG) induces mycobacterium-specific Interferon γ (IFN-γ) expressing CD4 and CD8 T dells that secrete Interferon γ (IFN-γ) when recognizing infected cells. IFN-γ in turn activates effector mechanisms in macrophages that kill or control the growth of the intracellular bacilli.

T cells are highly plastic regarding their fate: they can differentiate in different ways when their interacting innate immune cells produce fate changing cytokines in response to different pathogens and/ or diverse environmental stressors. There are several transcription factors like Signal Transducers and Activators of Transcription (STATs) that drive the differentiation "choice" or fate and control responses of T cells by directly manipulating the genes involved in T cell differentiation and functions. Less studied in the context of *M. tuberculosis* are the Hypoxia Inducible Factors (HIFs), which are central for the regulation of the cellular metabolism in adaptation to hypoxia. HIFs have been shown to be a major controller of immune responses against viruses and tumors.

Thus, the generation of mycobacteria-specific T cell responses, the immune mechanisms involved in the TB and DM comorbidity, as well as HIF-1 and its role in TB immunity are discussed in this thesis.

1.1 TUBERCULOSIS

TB (tuberculosis) was identified as an infectious disease and *M. tuberculosis* as the bacillus causing TB by Robert Koch in 1882. TB is an ancient infectious disease as archaeological studies indicate that prehistoric humans had TB as early as 4000 BC (Zink et al., 2003). It is stated: *´´It is impossible to know exactly how many people were infected by the white plague because "consumption" was used to describe any number of chronic lung disorders and many non-pulmonary forms of the disease were largely misdiagnosed. But by the estimate of some medical historians, as many as half of the population in early 19th century England died from tuberculosis. These included some of Britain's most famous literary icons: Lord Byron, Percy Shelly, John Keats, and Brönte sisters Maria, Elizabeth and Emily. The disease claimed the lives of Frederic Chopin and Anton Chekov, and became the subject of Giacomo Puccini's´´* (*The White Plague in the City of Angels*, n.d.) In Europe, *M. tuberculosis* infected probably 100% of the population and caused nearly 25% of all deaths during the white plague in the XVIII and XIX centuries (Barberis et al., 2017). While mortality has decreased dramatically, in 2018, an estimated 10.5 million individuals developed active TB, and TB caused about 1.5 million deaths. TB is the leading single infection bacterial killer in the world (*WHO | Global Tuberculosis Report 2019*, n.d.).

Patients with active TB will transmit *M. tuberculosis* to healthy individuals via aerosol droplets. While some of the infected individuals can clear/ sterilize the infection, many of the individuals infected with *M. tuberculosis* develop a chronic asymptomatic infection, also called latent TB infection (LTBI), and a third group around 5% of infected individuals will develop primary TB. LTBI may progress to active, symptomatic TB, usually affecting the lung (Drain et al., 2018; Salgame et al., 2015). In around 40% of the active TB cases, the disease can also affect other organs. Extrapulmonary TB occurs more commonly in immunosuppressed persons and young children, and affects the bones, the central nervous system, the draining or nearby lymph nodes, the pleura or the genitourinary systems (J. Y. Lee, 2015). Extrapulmonary TB results in systemic dissemination into single organs or causes a miliary TB with wide multiorgan disease and a high mortality rate. The co-infection with human immunodeficiency virus (HIV) and comorbidity with DM favor development of active TB.

BCG, the actual vaccine against TB was developed from attenuation due to multiple passages of *Mycobacterium bovis* and introduced in 1921. However, BCG has variable and incomplete efficacy in adults even though it protects children from disseminated TB. BCG remains the most widely used vaccine globally (Bloom et al., 2017), and new vaccines must be more efficient as BCG. Another challenge for control of *M. tuberculosis* infection is the emergence of resistant, multidrug-resistant and extremely drug-resistant strains of *M. tuberculosis*. (Allué-Guardia et al., 2021)

A granuloma, the hallmark of TB, is the environment in which the bacilli can survive but also the dissemination can be controlled. It is a well-organized histopathological structure present both in the lungs of active and latent TB individuals, mainly containing macrophages, epithelioid cells and Langhans giant cells surrounded by T cells (Silva Miranda et al., 2012).

Caseous necrosis or cheese like granulomas are classical histopathological features of human TB. The sarcoid or necrotic TB granulomas can be encapsulated by a collagen capsule produced by a fibroblast ring and become hypoxic (Harper et al., 2012). The dissemination of *M. tuberculosis* into distal sites in the lung, the adjacent lymph nodes and other systemic organs can occur if the granuloma is damaged and thereby mycobacteria reach blood vessels or the alveolar or bronchiolar lumen. During latent infection, calcified granulomas may develop from cellular or caseous granulomas. There are less inflammatory cells in this granuloma type which show a successful immune response and can be sterile. There are also some other granuloma types like non-necrotizing granulomas, necrotic neutrophilic granulomas and completely fibrotic granulomas (Flynn et al., 2011).

The main function of granuloma is to contain and control the dissemination of *M. tuberculosis*. *M. tuberculosis* can survive in the granuloma, due to a multitude of strategies to evade the immune responses and stress within the granuloma, developing into a latent or dormant stage. However, if the immune control wanes and thereby the granuloma containment is breached, *M. tuberculosis* proliferation reactivates leading to *M. tuberculosis* dissemination throughout the body or transmission. Whether *M. tuberculosis* is controlled or can disseminate depends on the immune microenvironment in granuloma composed by the cells forming the granuloma and present soluble mediators such as cytokines and chemokines (Guirado & Schlesinger, 2013).

1.2 *M. TUBERCULOSIS*

M. tuberculosis is an intracellular, rod-shaped, and acid-fast bacilli within the order Actinomycetales with 1-4 μm in length and 0.3-0.6 μm in width. *M. tuberculosis* has a generation time of around 20 h and belongs to the slow-growing mycobacterial species (Koch & Mizrahi, 2018). During the dormant state, *M. tuberculosis* cannot replicate but they are still viable, with minimal metabolic activity. This strategy allows the bacilli to be resistant to host defense and drug treatments. *M. tuberculosis* bacilli can be reactivated and develop into pulmonary TB eventually under favorable conditions like the presence of rich nutrients or in a compromised immune system (Peddireddy et al., 2017). *M. tuberculosis* is regarded as acid-fast bacteria with a waxy cell wall resistant to decolorization by ethanol/acids (Ziehl-Neelsen stain). A dominant feature of *M. tuberculosis* is the abundance of glycolipids in the cell envelope, constituting up to 40% of its dry weight and 60% of its cell wall. Peptidoglycan, arabinogalactan, mycolic acids, phosphatidylinositol mannosides, phthiocerol dimycocerosates, phenolic glycolipids, trehalose dimycolate and mannose-capped lipoarabinomannan all form this thick lipophilic membrane organized via either covalent or noncovalent binding (Kinsella et al., 2021). These lipids contribute to many biological properties of it including less permeability and resistance to various antibiotics which can protect it from hostile environments (Chiaradia et al., 2017; Cook et al., 2009). For example, mycolic acid, a main component of outer the cell membrane, is critical for *M. tuberculosis* virulence, viability, and induction of host immune responses.

1.3 MACROPHAGES

Macrophages, derived from the yolk sac, fetal liver and bone marrow in adults are present in lymphoid and most non-lymphoid tissues including lung, spleen, skin, liver, brain and heart. The tissue resident macrophages are named differently by their distribution (Varol et al., 2015). The macrophages in the lower lung located on the luminal surface of the alveolar space are called alveolar macrophage and are maintained by proliferation in the resident tissue. Macrophages can also be derived from monocytes recruited during inflammation after M-CSF differentiation (Geissmann et al., 2010). Macrophages are central in tissue remodeling, homeostasis, antigen presentation, and phagocytosis of pathogens, debris and dead cells. Macrophages have been divided into M1 macrophages with pro-inflammatory function and M2 macrophages with anti-inflammatory function, but this classification -although helped to understand their biology- has been shown to be an oversimplification when considering the variety of macrophages and their features and functions. The M1 macrophages are polarized under T_H1 cytokines especially IFN-γ or by lipopolysaccharide (LPS) recognition and can produce cytokines like tumor necrosis factor (TNF), interleukin (IL)-1α, IL-1β, IL-12 and perform antimicrobial functions against intracellular bacteria and protozoa. These activated macrophages express a broad range of effector molecules including reactive oxygen and nitrogen species and promote phagolysosome maturation and acidification. Instead, M2 macrophages are induced by T_H2 cytokines such as IL-4, IL-13, IL-10, IL-21, IL-33 and secrete IL-10, transforming growth factor-β (TGF-β), and have potent phagocytosis capability and help in tissue repair and have anti-inflammatory features (Shapouri-Moghaddam et al., 2018).

Alveolar macrophages (AM) and interstitial macrophages regulate homeostasis and inflammation or infection in the lung. Tissue-resident AMs are the first line of defense against pathogens and pollutants in the lung (F. Hou et al., 2021). Normally, AMs are poor at antigen presentation but can transport antigens to the draining lymph nodes (dLN). In healthy individuals, they secret prostaglandins and TGF-β to suppress T cell activation and promote the development of regulatory T cells (Treg cells) preventing inflammatory responses (Hussell & Bell, 2014). The phagocytic ability of resident AMs is substantially increased through interaction of receptors in the AM with pathogen-associated molecular patterns when there is an infection or inflammation. AMs may subsequently transport pathogens to the lung dLN (Kopf et al., 2015). During infection and inflammation, AM will recruit monocytes via the secretion of CCL2. The $CCR2^+$ monocyte will differentiate into inflammatory macrophages that are better equipped for killing microbes and controlling the infection than the AMs (F. P. Martin et al., 2021).

1.4 T CELLS

T cells mediate immune responses against pathogens of all types, allergens and tumors. Lymphoid progenitors derived from bone marrow can populate thymus, where they further differentiate to mature T cells (B. V. Kumar et al., 2018). The thymocytes become mature after undergoing a series of well-documented differentiation steps and ultimately positively and negatively selected, self-tolerant T cells emigrate from the thymus and seed the peripheral

secondary lymphoid organs (Zúñiga-Pflücker, 2004). The mature naïve T cells express distinct types of antigen receptors and recirculate in the body. These naïve T cells are activated by recognizing antigens that are presented by major histocompatibility complex (MHC) molecules on antigen-presenting cells (APCs) recognized via their T-cell receptor (TCR), if such recognition is in parallel accompanied by a co-stimulatory signal, provided by the co-stimulatory receptor after binding to their ligands on dendritic cells (DCs). The TCR and co-stimulatory receptor engagement leads to signaling cascades that include several kinases and results T cell activation, with important metabolic activation, size growth and proliferation required for clonal expansion (Hwang et al., 2020), resulting in effector T cells. The activated antigen-specific T cells differentiate into different effector subsets with diverse functions depending on the type of infection and environment (including cytokines). Most activated T cells perform their effector functions and will then die by apoptosis when the pathogen is eliminated. However, some activated effector T cells will further differentiate into memory cells that may reside in lymph nodes or in the peripheral tissues. Memory T cells will mount faster and stronger responses upon re-encountering the same infection (Cuddapah et al., 2010).

1.4.1 T cell activation

The T cell activation is a crucial process in the adaptive immune response initiating numerous signaling cascades that ultimately determine the cell's fate such as differentiation and survival. A simplified TCR signaling pathway explaining how a T cell is activated is shown in figure 1 (Pollizzi & Powell, 2014). There are three signals required for complete activation of naïve T cells by APCs. These three signals are binding of MHC-antigen complex by TCR complex and co-receptors, co-stimulatory signals for example delivered by the CD28 receptor after binding CD80/ 86 on the DCs and the presence of cytokines such as IL-12, IL-4 or IL-23 required for T cell differentiation (Kagoya et al., 2018).

T cells specifically recognize antigen on MHC presented by APCs via their TCR complex composed of TCR and CD3 (Xu et al., 2020). Engagement of a T cell with APCs induces the formation of an immunological synapse which provides a stable platform and sustainment for TCR signaling transduction and the generation of effector T cells (Colin-York et al., 2019). The ligation of the co-receptor CD4 or CD8 with MHC molecules leads to the phosphorylation of tyrosines in the immunoreceptor tyrosine-based activation motifs (ITAMs) on CD3 cytoplasmic domains by the Src kinase Lck followed by initiation of intracellular signaling transduction (Guy et al., 2013). The Zeta-chain-associated protein kinase 70, a tyrosine kinase, will be then recruited to the phosphorylated ITAMs and be itself also phosphorylated by Lck. ZAP70 will in turn induce the phosphorylation of the transmembrane adapter molecule linker for activation of T cells (LAT) and leukocyte phosphoprotein of 76 kDa (SLP-76) (Oniszczuk et al., 2020). The LAT signalosomes formed above will provide the SH2- and SH3- binding sites for downstream targets such as phospholipase C (PLC)-γ1 (Saveanu et al., 2019). The PLC-γ1 cleaves phosphatidylinositol bisphosphate into diacylglycerol and IP3 which binds IP3 receptors on endoplasmic reticulum (ER) inducing Ca^{2+} release from ER. The Ca^{2+} depletion in the ER is sensed by stromal interaction molecules resulting in the activation of calcium

channels and the Ca^{2+} influx across the cell surface membrane (Trebak & Kinet, 2019). Ca^{2+} in the cytosol binds to the cofactor calmodulin and activates the calcineurin phosphatase. The activated calcineurin complex will dephosphorylate the nuclear factor of activated T-cells (NFAT), a transcription factor that induces the generation of the cytokine IL-2 which is required for T cell proliferation in an autocrine fashion (Srikanth & Gwack, 2013).

The costimulatory receptor CD28 is essential for the initiation and regulation of signaling transduction following TCR engagement of naïve T cells. CD28 ligation activates the PI3K /AKT /mTOR pathway and the subsequent activation of HIF-1 and MYC for T cell metabolism figure 1 (Bhattacharyya & Feng, 2020). Crosstalk between mTOR and MYC induces metabolic reprograming such as glycolysis and glutaminolysis in naïve T cells after TCR engagement. The metabolic shift increases the uptake of glucose, amino acids and other nutrients that are required for T cell growth and proliferation (Waickman & Powell, 2012). Without CD28 involved in T cell costimulation, anergy is induced in T cells leading to T cells that are functionally inactive or unresponsive and ultimately towards cell death (June et al., 1990). In T cells primed without CD28 ligation, NFAT is activated but MAPK signaling, and transcription factor AP-1 are repressed leading to reduced protein synthesis, cell cycle, proliferation, and IL-2 production. Addition of IL-2 could restore impaired proliferation caused by absence of CD28 (ElTanbouly & Noelle, 2021).

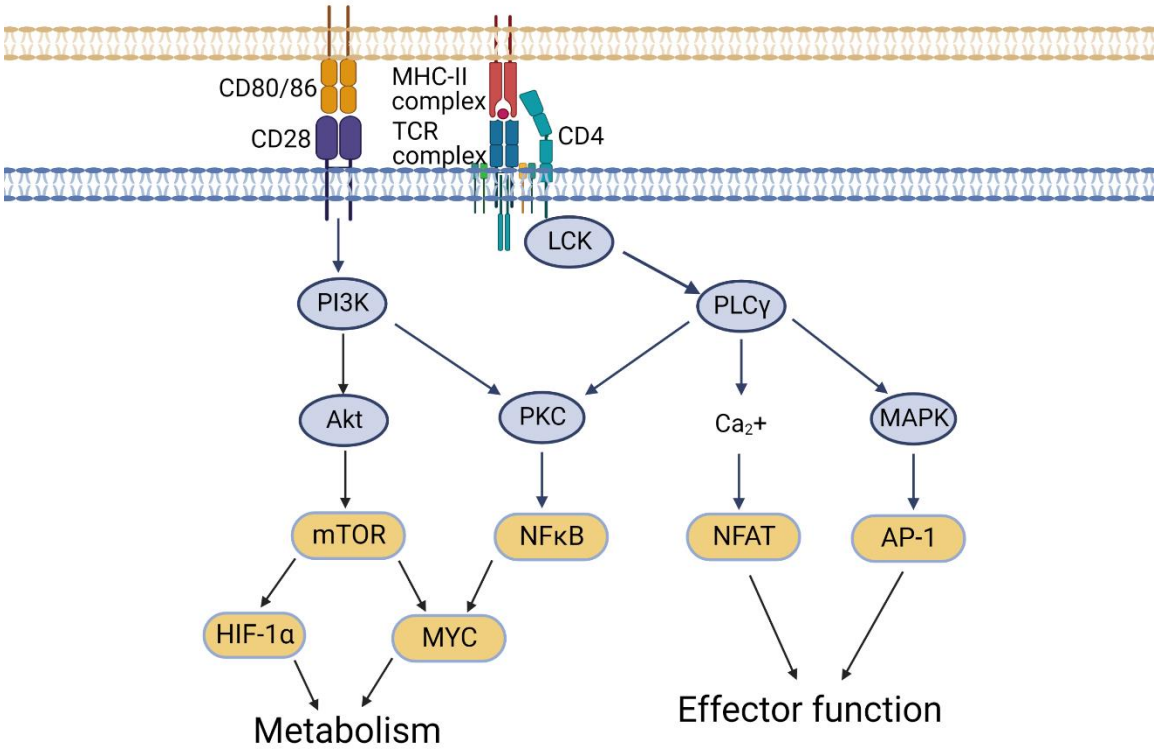

Figure 1: **TCR signaling pathway.** *Created with Biorender.com.*

1.4.2 T cell differentiation

Naïve T cells are activated in the lymph nodes when they recognize antigens presented by MHC II on DCs via T cell receptor engagement. CD4 and CD8 T cells make up most T cells and recognize peptide-MHC II complex and peptide-MHC I complex during T cell activation respectively due to their co-receptors of CD4 and CD8 (Luckheeram et al., 2012). Basically, CD4 T cells help B cell responses, cytotoxic CD8 T cells and macrophages by producing various cytokines and can be important for the suppression of immune responses (Luckheeram et al., 2012). T cells can differentiate into different T cell subsets characterized by different transcription factors under certain cytokine environments. CD4 T cells may for example develop into T helper (T_H)1, T_H2, T_H17, T follicular helper (T_{FH}) cells and Treg cells. The JAK/ STAT is the primary signaling pathway triggered by cytokines. The STAT transcription factors are coordinated with other transcription factors involved in the T cell fate determination, differentiation and cytokine production as shown in figure 2 (Liudahl & Coussens, 2018; Tripathi & Lahesmaa, 2014). Naïve CD8 T cells differentiate into cytotoxic CD8 T cells after activation and carry out the function through cytotoxicity and cytokines production that play an important role in virus infections and cancer (Kaech et al., 2002). The differentiated T cell subsets play critical roles in the responses against a wide variety of pathogens and tumor immunity but also regulate autoimmune and allergic responses.

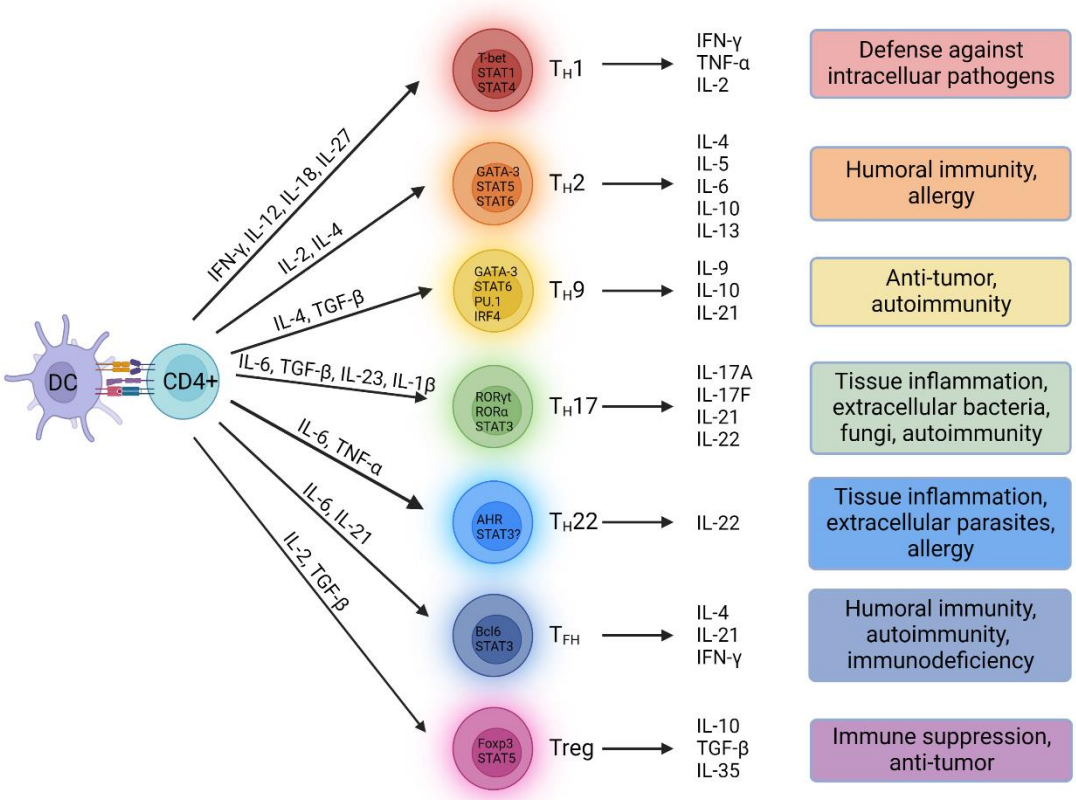

Fig 2: **CD4 T cell differentiation.** *Created with Biorender.com.*

Memory T cells, staying in the body after the clearance of a pathogen, are mainly subdivided into central memory (T_{CM}), effector memory (T_{EM}) and resident memory (T_{RM}) T cells according to their spatial distribution and circulation (Schenkel & Masopust, 2014) (figure 3). These cells respond rapidly to pathogen re-exposure and can provide life-long protection against infections (Farber et al., 2014). T_{RM}, located in non-lymphoid tissues such as skin, lung and intestinal mucosa, confer local protection as earliest T cell population after recognizing antigens (Chang et al., 2014; Nguyen et al., 2019). T_{RM} highly express CD69, a membrane-bound type II C lectin receptor, together with CD103 in many cases. These cells derived from Killer Cell Lectin Like Receptor G1 $(KLRG1)^{low}$ and C-X-C chemokine receptor (CXCR) 3^{+} effector cells and require TGF-β, TNF and IL-15 for their differentiation, migration and maintenance (Mueller & Mackay, 2016; Nguyen et al., 2019). The early effector subset $CD127^{-}CD62L^{hi}CD27^{+}$ observed in chronic parasite infection may differentiate into T_{EM} and T_{CM} after the contraction phase of the immune response (Opata et al., 2015). T_{EM} circulating in blood are characterized by rapidly turning on effector functions after migration into the inflammatory sites, producing cytokines like IFN-γ and TNF-α (Muroyama & Wherry, 2021; Sallusto et al., 2004).

T_{CM}, primally found in lymph nodes, secret more IL-2 and have a high proliferative and differential ability upon restimulation (Busch et al., 2016; Kallies, 2008). The chemokine receptor CCR7 controls their homing of immune cells to secondary lymphoid organs via its ligands CCL19 and CCL21 (Förster et al., 2008; Sallusto et al., 1999). Although expression of CD45 isoforms can only be used for discrimination between human naïve and memory cells, $CD44^{low}$ $CCR7^{+}$ $CD62L^{high}$ distinguish naïve T cells, $CD44^{high}$ $CCR7^{+}$ $CD62L^{high}$ T_{RM}, and $CD44^{high}$ $CCR7^{-}$ $CD62L^{low}$ T_{EM} in both mouse and human T cells (Raphael et al., 2020).

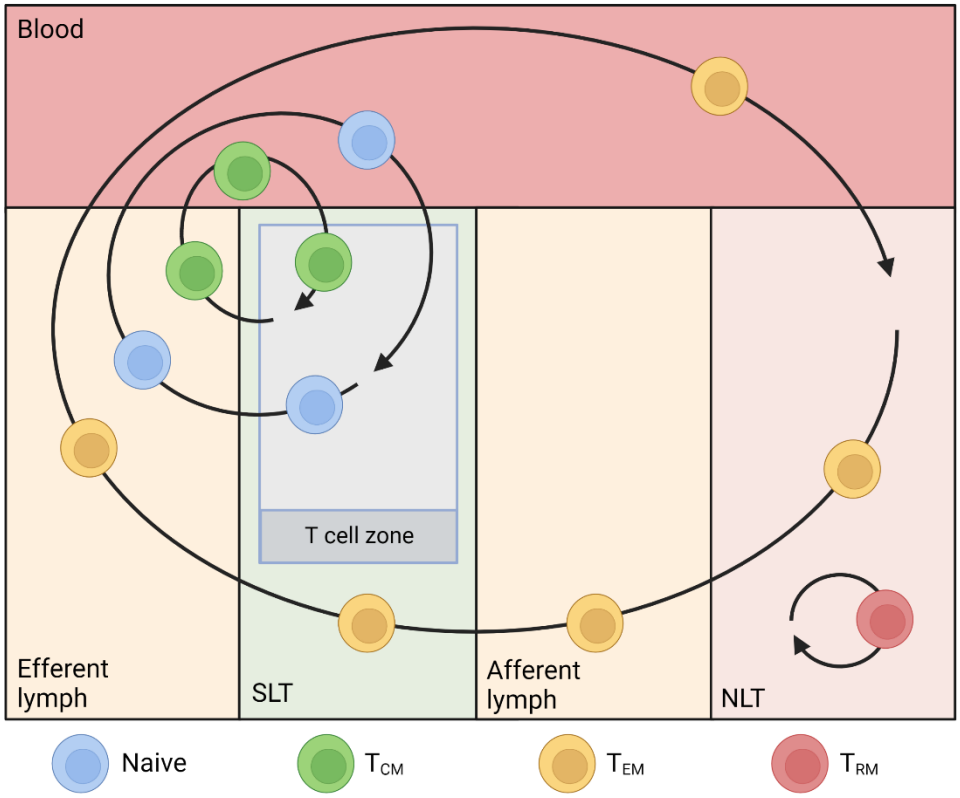

Figure 3: **Naïve and memory T cell migration patterns.** (Inspired by (Schenkel & Masopust, 2014) *Created with Biorender.com.* SLT: secondary lymphoid tissues, NLT: nonlymphoid tissues

In the lymph nodes, activated CD8 T cells receive signals from pro-inflammatory cytokines produced during infection after antigen priming. Those cytokines will activate signaling via diverse transcription factors such as T-bet, Eomesodermin, blimp-1, bcl-6, id-2 and 3 and STAT-3, -4, -5 or -6 to shape CD8 T cell differentiation and proliferation (Kaech & Cui, 2012). Then the effector cytotoxic T lymphocytes (CTL) migrate to infection site and will release cytokines, perforin, granzymes to target infected cells for apoptosis. $KLRG1^{+}$ effector CD8 T cells that survive the effector-to-memory transition will give rise to T_{EM}. These are prominent in peripheral tissues in mice models of infection (Cui & Kaech, 2010). CTLs also secrete effector cytokines such as IFN-γ and TNF contributing to host defenses. Chemokine receptor CX3C motif chemokine receptor 1 (CX3CR1) is used to define effector and memory CD8 T cells during virus infection (Gerlach et al., 2016). Similar as in CD4 T cells, CD8 T_{RM} are $CD103^{hi/lo}CD69^{hi/lo}CD127^{int}$, CD8 T_{EM} are $CCR7^{lo}CD62L^{lo}CX3CR1^{hi}CD127^{hi}$ and CD8 T_{CM} are $CCR7^{hi}CD62L^{hi}CX3CR1^{lo}CD127^{hi}$ (Omilusik & Goldrath, 2019). Memory T cell differentiation is briefly summarized as shown in figure 4 (Raphael et al., 2020; Šustić et al., 2021).

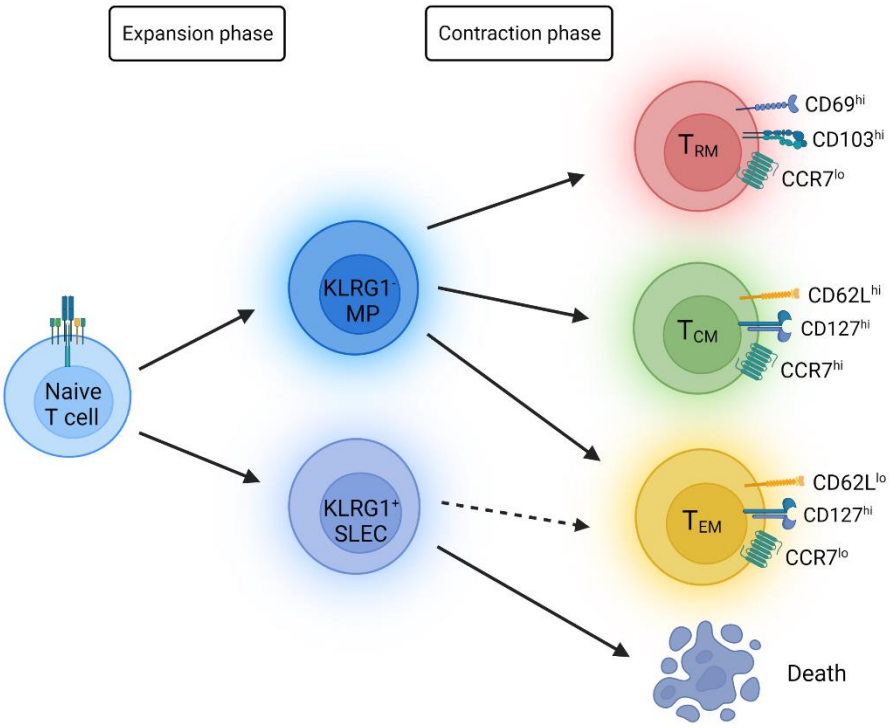

Figure 4: **Memory T cell differentiation.** *Created with Biorender.com.*

1.5 IMMUNITY TO *M. TUBERCULOSIS*

1.5.1 Innate immune responses to *M. tuberculosis*

The infection with *M. tuberculosis* occurs by inhalation of aerosol droplets containing tubercle bacilli from an infectious person. Aerosols are mainly generated by coughing but also by sneezing, shouting or singing. The inhaled bacilli reach the alveoli and are phagocytized by the luminal alveolar macrophages (Sakamoto, 2012).

Macrophages recognize *M. tuberculosis* through pattern recognition receptors (PRRs) resulting in downstream signaling activation. The PRRs include Toll-like-, Nod-like-, C-type lectin-, mannose-, scavenger- and complement receptor families. A cascade of signaling molecules resulting in cell activation and the secretion of cytokines and chemokines are induced during this process. The former will recruit monocytes, neutrophils and T cells to the infected sites and will form a in a well-organized lesion, the granuloma (Saiga et al., 2011).

Apoptosis is a type of cell death with cellular deconstruction resulting in the formation of and release of apoptotic bodies. The apoptotic bodies can be engulfed by phagocytes expressing "eat me" signals on the cell surface (J. Lee et al., 2009). This process is also called efferocytosis and has been reported important for host defense against *M. tuberculosis* infection. *M. tuberculosis*-infected macrophages can become apoptotic leading to uptake of apoptotic bodies containing bacteria by uninfected macrophages both in vitro and in vivo experiments. *M. tuberculosis*-mediated apoptosis can be induced by TNF for the extrinsic apoptotic pathway and by mitochondrial outer membrane permeabilization for the intrinsic apoptotic pathway with both pathways leading to caspase-3 activation (Behar et al., 2011). Efferocytosis restricts *M. tuberculosis* growth by promoting phagosome maturation and delivery of bacilli into the lysosomal compartment (C. J. Martin et al., 2012). However, during *M. tuberculosis* infection, necrosis instead of apoptosis usually takes place, a process which impairs bacillary control. Infections with virulent *M. tuberculosis* strains induce lipoxin A4 in infected macrophages that promotes necrosis rather than prostaglandin E2 which protects against necrosis in general taking place after infection with attenuated mycobacteria (Behar et al., 2010). Recently, Seungwha showed that a mycobacterial acyl carrier protein inhibits host cell apoptosis via reducing reactive oxygen species generation and activation of c-Jun N-terminal kinase which in turn enhances the intracellular survival of *M. tuberculosis* (Paik et al., 2019). Thus, if *M. tuberculosis*-infected alveolar macrophages migrate into the lung and recruit uninfected macrophages, this promotes the transfer and dissemination of bacilli.

CD4 T cell mediated responses are central in the control of *M. tuberculosis* as shown by the increased risk of developing TB in HIV infected individuals. IFN-γ produced by T cells is also required for macrophages to display their full antimicrobial functions during *M. tuberculosis* infection (figure 5) (Bonecini-Almeida et al., 1998; Shanmuganathan et al., 2022). IFN-γ mediates mycobacterial killing by stimulating the production of reactive nitrogen and oxygen intermediates (RNIs and ROIs), phagosome maturation, acidification and fusion of the phagosome with the lysosome. RNIs and ROI further damage *M. tuberculosis* (P. Kumar,

2017). Other innate cytokines, including TNF-α, IL1β, granulocyte-macrophage colony-stimulating factor (GM-CSF), are also important for macrophage activation restricting the growth of *M. tuberculosis* (Bryson et al., 2019; McKell et al., 2021).

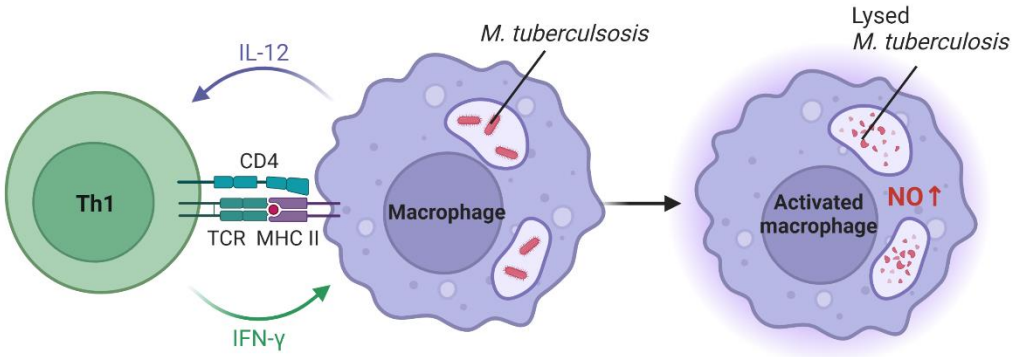

Figure 5: **IFN-γ mediated *M. tuberculosis* killing by macrophages.** *Created with Biorender.com.*

Other innate immune cells, that may be involved in the establishment of *M. tuberculosis* infection, are DCs, neutrophils, natural killer cells and non-classical T cells (C. H. Liu et al., 2017; Ravesloot-Chávez et al., 2021).

M. tuberculosis has developed complex mechanisms to evade these broad protective immune responses described. For example, and among many mechanisms described, *M. tuberculosis* secretes proteins via the ESX secretion system that can alter host anti-bacterial mechanisms. It was reported that *M. tuberculosis* PtpA, a secreted tyrosine phosphatase, could suppress innate immune responses via binding to host ubiquitin leading to inhibition of NF-κB and cytokine production, ESAT-6 might inhibit TLR2 signaling and MHC-I presentation and PtpB can suppress macrophage apoptosis and the secretion of IL-1β (Chai et al., 2020; Pathak et al., 2007; Sreejit et al., 2014; J. Wang et al., 2015). Iron (Fe) is a vital micronutrient for essential biological processes in both the host and *M. tuberculosis*. Dysregulated Fe metabolism in hematopoietic stem cells by IFN-I signaling induced by *M. tuberculosis* infection results in limited myelopoiesis, impaired trained immunity and *M. tuberculosis* control (Khan et al., 2020). Besides, evasion mechanisms of *M. tuberculosis* involve impaired phagosome maturation, suppression of phagolysosome fusion, inhibition or a delay of antigen presentation and thereby T cell activation (Ernst, 2018).

1.5.2 CD4 T cells in TB

As indicated above, CD4 T cells are critical for control of the infection with *M. tuberculosis*, which is reflected in the increased risk of developing TB of patients co-infected with HIV when CD4 cells drop in numbers (Prezzemolo et al., 2014). In humans, CD4 and CD8 T cell

responses to *M. tuberculosis* can be detected three to eight weeks after infection (Jasenosky et al., 2015). Naïve T cells are activated in the mediastinal lymph node through the recognition of antigens presented by DCs and then migrate to lung. The main function of CD4 T cells during *M. tuberculosis* infection is predominantly carried out by secreting a variety of cytokines to activate macrophages or to attract other immune cells to the site of infection. T_H1 cells secrete IFN-γ which in turn activates macrophages that perform antimicrobial functions. The importance of IFN-γ in *M. tuberculosis* infection is demonstrated in children with mutations in the IFN-γ receptor 1, IL-12, IL-12R and STAT1 who have an increased mendelian susceptibility to infection with otherwise nonpathogenic, opportunistic mycobacteria (Holland, 2007). Differentiation and maintenance of T_H1 and IFN-γ secretion by CD4 T cells is dependent on IL-12, secreted by *M. tuberculosis*-activated DCs (O'Garra et al., 2013). The level of IFN-γ secreted by CD4 T cells is regulated by multiple pathways resulting in different outcomes of *M. tuberculosis* infection. Receptors on myeloid cells are well known to play an important role in IFN-γ expression control T_H1 cells increasing or suppressing such secretion (J. Li et al., 2020; X. Li et al., 2021).

However, although IFN- γ is required for protection, IFN-γ levels are not necessarily correlated with protection against *M. tuberculosis* infection (Abebe, 2012). For example, although intravascular effector cells, which are $CX3CR1^+$, produce more IFN-γ than lung parenchymal $CXCR3^+$CD4 T cells, they show a lower protective capability (Sakai et al., 2014). In addition, CD4 T cells secretion of IFN-γ is suppressed by the inhibitory receptor PD-1 (Sakai et al., 2016). Instead, the levels of $CXCR3^+$ CD4 T cells are inversely correlated with the bacterial burden in the lungs from nonhuman macaques during LTBI (Shanmugasundaram et al., 2020.). The parenchymal location of $PD1^+CXCR3^+$CD4 T cells and their ability to divide accounts for the ability to transfer protection against *M. tuberculosis* infection (Moguche et al., 2015) IFN-γ production within the granuloma is shown to be inhibited by TGF-β. In turn, the deletion of the TGF-β receptor in T cells resulted in increased IFN-γ levels (Gern et al., 2021).

Besides IFN- γ, other cytokines such as IL-17 and TNF also play an important role in the control of *M. tuberculosis* infection. IL-17 production by T_H17 CD4 T cells is induced by IL-23 during a mycobacterial infection contributing to the recruitment of neutrophils (Curtis & Way, 2009). However, excessive IL-17 levels can result in exacerbated inflammation and tissue damage. Recently, a population of CD4 T cells named T_H1 /T_H17 cells has been described during *M. tuberculosis* infection. Those cells coexpress T-bet and RORγt, coproduce IFN-γ and IL-17, and express CXCR3 and CCR6 (Shanmugasundaram et al., 2020.).

TNF production can be induced in T cells, macrophages and DCs and is involved in immune and pathologic responses during *M. tuberculosis* infection. One function of TNF is to synergize with IFN-γ activating nitric oxide synthase 2 expression, which further induces the production of nitric oxide (NO) and RNIs for antimycobacterial effects of macrophages (Flynn & Chan, 2001). TNF is central for protection against *M. tuberculosis* and required for granuloma formation.

Treg cells might help in the establishment of the *M. tuberculosis* infection in the lungs by hampering protective responses. For instance, IL-10 produced by Treg cells regulates T cell immune responses to *M. tuberculosis* via inhibition of mainly T_H1 and T_H17 cell functions thereby promoting bacterial growth (Jasenosky et al., 2015). The onset of T cell responses against *M. tuberculosis* infection in mice (that is detectable around 3 weeks post infection) is delayed as compared to other infections, probably via regulation of Treg cells (Urdahl et al., 2011). On the contrary, low Treg cell levels may facilitate excessive inflammatory responses, suggesting that the balance between Treg cells and TB development is very important (Cardona & Cardona, 2019).

1.5.3 CD8 T cells in TB

Mouse studies initially suggested a protective role for CD8 T cells during *M. tuberculosis* infection, but at least in the mouse experimental infection their relevance is lower than that of CD4 T cells (Prezzemolo et al., 2014). Mycobacteria-specific CD8 T cell responses are generated during *M. tuberculosis* infection in humans and are characterized by the production of IFN-γ and TNF (Lin & Flynn, 2015). In addition, cytotoxic granules of human CD8 T cells contain granulysin, a molecule that kills *M.* tuberculosis through pores formed on target cells by perforin. After help from CD4 T cells, CD8 T cells display an increased ability of producing cytokines, a reduced expression of inhibitory receptors and an improved mycobacterial control. Although CD8 T cells are present within the granuloma, they show a reduced granulysin and perforin content at that site (Andersson et al., 2007). In contrast, depletion of CD8 T cells in a macaque model resulted in a deficient BCG-induced protection against *M. tuberculosis* (C. Y. Chen et al., 2009).

Many studies show that *M. tuberculosis* can perturb the initiation of adaptive immunity by multiple mechanisms. Alissa *et al.* showed that DCs migration to dLN could be postponed by *M. tuberculosis*, contributing to a less efficient T priming (Chackerian et al., 2002). T cell responses to *M. tuberculosis* can also be inhibited by lipoxin A4 produced by *M. tuberculosis*-infected macrophages inhibiting apoptosis, an indispensable step to present *M. tuberculosis* antigens and to promote CD4 and CD8 T cell responses (Larson et al., 2013; Urdahl et al., 2011). *M. tuberculosis* EsxH affects CD4 T-cell priming and proliferation thorough impairing antigen presentation in macrophages and DCs (Portal-Celhay et al., 2016). A natural polymorphism of in the EsxH gene was also associated with reduced CD8 T cell priming (Sutiwisesak et al., 2020).

Additionally, the T cell differentiation and effector functions can be altered by immune evasion mechanisms of *M. tuberculosis*. *M. tuberculosis* surface lipids can regulate the expression of the costimulatory molecule CD28 as well as CD40L on T cells repressing the polarization of T_H1 and T_H17 cells (Ankley et al., 2020). Antigen specific CD4 T cells express high levels of markers associated with exhaustion such as PD-1, LAG 3, TIM3 and 2B4 during chronic infection (Behar et al., 2014). However, PD-1^+ T cells can further proliferate and differentiate into terminally differentiated effector KLRG1^+ T cells with high levels of cytokine production (Hu et al., 2018), suggesting the importance of the PD-1^+ T cell population during *M.*

tuberculosis infection as the one that maintains T cell memory. In macaques, PD-1 blockade leads to reduced CD4 T cell responses and exacerbated *M. tuberculosis* infection (Kauffman et al., 2021). The protective role of PD-1^+ T cells in cytokine production and proliferation of CD4 T cells has been further demonstrated in TB patients (Day et al., 2018). This is in contrast to a study using peripheral blood mononuclear cells from pulmonary TB patients, in which blockage of the PD-1 pathway restored IFN-γ and IL-2 production by T cells stimulated with *M. tuberculosis* antigens (Singh et al., 2013).

1.5.4 T_{RM} in TB

The accumulation of T_{RM} in mucosal sites has been suggested as a first line of defense against pathogens that re-enter the body (Schenkel & Masopust, 2014). The role of *M. tuberculosis*-specific T_{RM} in mice was first reported in 2014. The adoptive transfer of *M. tuberculosis* -specific T_{RM} to T cell deficient mice could reconstitute the parenchyma and result in potent protective immune responses against *M. tuberculosis* infection (Hu et al., 2018; Sakai et al., 2014). BCG, the only licensed TB vaccine, can induce expansion of mycobacteria-specific T_{RM} in mice. As the expansion of mycobacteria-specific T_{RM} in the lungs is associated with different BCG administration strategies, those could determine the efficacy of the vaccine. Compared to subcutaneous BCG vaccination, mucosal BCG vaccination leads to higher levels of protection against *M. tuberculosis* infection in mice and generates a greater number of *M. tuberculosis*-specific T_{RM} in the lung (Perdomo et al., 2016). The importance of T_{RM} in *M. tuberculosis* -infected mice is further indicated in the study on previously immunized mice that were additionally treated with FTY720, the sphingosine-1-phosphate (S1P) receptor agonist. FTY720 could prevent T cells from circulating but had no effect on the percentage of *M. tuberculosis* -specific T_{RM} in the lung of immunized mice. The FTY720-treated immunized mice conferred equal protection against *M. tuberculosis* infection compared to untreated immunized mice, suggesting that the accumulation of lung-resident memory T cells following vaccination was sufficient to protect mice during *M. tuberculosis* infection (Flórido et al., 2018).

1.6 RESPONSES TO HYPOXIA OF MAMMALIAN CELLS

1.6.1 Hypoxia inducible factors

The supply with oxygen is crucial for tissue function, cellular metabolism and homeostasis. Oxygen levels vary a lot in different tissues, with a 13% pressure of oxygen (PO_2) in the pulmonary vasculature and 1% PO_2 in the thymus (McNamee et al., 2013). Variations in the *P*O2 in the same tissue can be part of normal physiology, such as during exercise. Hypoxia is the state of insufficient oxygen availability to maintain homeostasis at the tissue level and is a pathological condition. Hypoxic tissue can be attributed to insufficient oxygen delivery as a consequence of low blood supply or deficient blood oxygen.

The HIFs are critical regulators of homeostasis of eukaryotic cells in response to hypoxia. HIFs will sense the low oxygen levels and allow cells to survive in such conditions. HIFs are heterodimeric transcription factors composed of an α and a β subunit. The HIF family is divided into 3 members HIF-1, HIF-2, and HIF-3, that are distinguished by three HIF-α isoforms: HIF-1α, HIF-2α, and HIF-3α will dimerize with HIF-1β, HIF-2β, and HIF-3β respectively (Yeo, 2019). HIF-1α was first described as an important sensor and regulator of oxygen-dependent transcriptional responses by Semenza *et al.* in 1995 (G. L. Wang et al., 1995). In general, HIFs activate glucose transporters and the enzymes involved in the glycolytic and carbohydrate pathways such as the pentose phosphate pathway. While the uptake of amino acids such as glutamate as well as fatty acids and acetate are enhanced by HIFs, the TCA cycle and fatty acid oxidation are hampered (de Heer et al., 2020). HIF-mediated metabolic changes allow the production of ATP in an oxygen-independent manner. HIFs also promote cell proliferation and survival (but may also be involved in apoptosis), the formation of blood vessels and the production of red blood cells, and the restoration of chronic wounds in diabetes, the regeneration of damaged tissues and the migration of keratinocytes.

Even the simplest invertebrates (such as the roundworm Caenorhabditis elegans) encode at least one single HIF, indicating a primordial function of HIF in mediating the adaptive responses that allow cells to survive oxygen deprivation (Luongo et al., 2017). Thus, HIF may have a very important role in the evolution of metazoa. Interestingly, HIFα is observed to have undergone multiple duplications in coincidence with the evolution of vertebrate species.

In this thesis, we basically focus on the role of HIF-1 regulating the metabolism and the function of immune responses.

HIF-1 is a heterodimeric transcription factor comprising HIF-1α and HIF-1β subunits (McNamee et al., 2013). HIF-1β, also known as aryl hydrocarbon receptor nuclear translocator (ARNT), is constitutively expressed within the nuclei regardless of oxygen availability. Both HIF-1α and HIF-1β belong to the basic helix-loop-helix-Per-ARNT-Sim (Bhlh-PAS) family. The global knockout of HIF-1α and HIF-1β resulted in embryonic lethality in mice (Ke & Costa, 2006). HIF-1β is promiscuous and dimerizes with other molecules besides HIF-1α forming numerous transcription factors such as AHR, neuronal PAS proteins and single-minded proteins (Wu et al., 2015).

Both HIF-1α and HIF-2α are hydroxylated in the proline residues by prolyl hydroxylases (PHDs) in normoxia (figure 6). The hydroxylated HIFs are then recognized by von-Hippel Lindau tumor suppressor (VHL), an E3 ubiquitin ligase targeting HIFs to degradation. The factor inhibiting HIF (FIH) can also hydroxylase HIF-1α, but via asparagine 803, resulting in a blockage of the interaction between HIF-1α and coactivator p300/CBP inhibiting nuclear translocation. However, during hypoxia, the initial hydroxylation does not occur and HIF-1α is thereby stabilized. HIF-1α dimerizes with HIF-1β after translocation into the nucleus. HIF-1 dimer binds to hypoxia-response elements in the promoter or enhancer sequences of certain target genes as shown in figure 6 (Palazon et al., 2014; Sunkari et al., 2015; Yang et al., 2020).

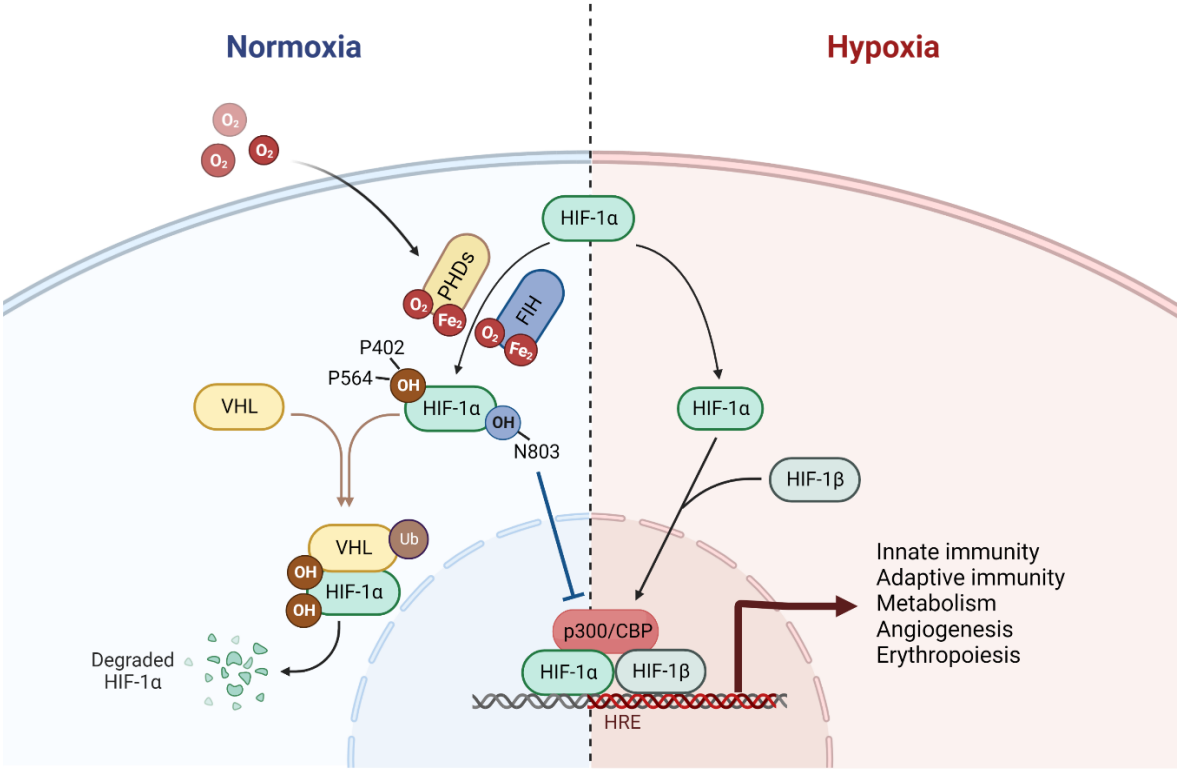

Figure 6: **Regulation of HIF-1α.** *Created with Biorender.com.*

HIF-1α is broadly expressed in nearly all embryonic cells and mediates the formation of blood vessels in embryos. Interestingly, HIF-2α is exclusively expressed by some cell types including epithelial cells, hepatocytes, macrophages, and astrocytes. (Keith et al., 2011). HIF-1α and HIF-2α are essential for the hypoxic responses of developing embryos. HIF-1 has been shown to control the expression of approximately 200 downstream genes that are mostly responsible for the glycolytic pathway. In contrast to HIF-1, HIF-2 promotes the expression of genes related to tumor growth, cell cycle and maintenance of stem cell (Loboda et al., 2010; Zhan et al., 2015). HIF-1 and HIF-2 share many downstream targets such as vascular endothelial growth factor (VEGF) but also have exclusive targets.

In contrast to HIF, MYC regulates mitochondrial respiration via mitochondrial biogenesis. HIF reduces the levels of MYC, partially dependent on upregulation of MYC antagonist MXI-1 (Sutphin et al., 2007). The expression of MXI-1 mRNA is directly controlled by HIF-1 and

HIF-2. MYC activity can be also downregulated by HIF-1, via downregulation of PGC-1β, a MYC target that promotes mitochondrial biogenesis (H. Zhang et al., 2007).

Expression of HIF-1α and HIF-2 α can be stabilized by inflammatory stimuli, cytokines and reactive oxygen species in normoxia, in a condition named pseudohypoxia such as when succinate accumulates, ketoglutarate is deficient, or iron is limiting for proper activity of the prolyl hydroxylases.

It has been well studied that HIFs are expressed upon immune cell activation and regulate responses of macrophages and T cells. In macrophages, HIF-1 has been demonstrated to enhance phagocytosis, bacterial killing, the production of NO and the secretion of proinflammatory cytokines (Peyssonnaux et al., 2005). In macrophages, HIF-2 stabilization has been shown to hamper oxidative phosphorylation and impair nitrosylation, thereby having opposite activity as compared to HIF-1 (Winning & Fandrey, 2022). In T cells, most studies have investigated the role of HIF-1. HIF-1 has been demonstrated to play an essential role in CD4 T cell differentiation and glycolysis, and in the CD8 T cell effector function and T cell survival (Y. Chen & Gaber, 2021). Studies revealed that HIF-2 is vital for the inhibition of Treg cell differentiation and the promotion of CD8 effector functions (Y. Chen & Gaber, 2021).

In T cells, T cell receptor (TCR) stimulation, cytokines and the metabolic checkpoint kinase the mammalian target of rapamycin (mTOR) have been shown to stabilize HIF-1α in normoxia (Doedens et al., 2013). The phosphoinositide-dependent kinase-1 and mTORC1 pathway also contribute to the induction of HIF-1α which is important for the glycolysis and effector functions in CD8 T cells (Finlay et al., 2012). Stabilization of HIF-1 by the lack of Vhl in T cells enhanced effector function of cytotoxic CD8 T cell during virus infection and mediated potent control of persistent virus infection (Doedens et al., 2013). TCR ligation in presence of cytokines activating STAT3 (such as IL-6) have been shown to induce the accumulation of HIF-1 and had consequences to T cell differentiation as described below (E. V. Dang et al., 2011).

1.6.2 VHL

The VHL gene was identified as a tumor suppressor in 1993 (Latif et al., 1993). The VHL protein is 24-30 kDa and contains 213 amino acid residues (Iliopoulos et al., 1995). VHL is a subunit of a Skp1-Cul1/Cdc53-F-box (SCF)-like E3 ubiquitin ligase complex that also includes elongin B and C (Kamura et al., 2000). Deficiency of VHL gene causes an autosomal dominant disorder leading to kidney tumors especially retinal carcinomas (Maxwell et al., 1999). Hypomorphic mutations in VHL also result in increased HIF-1α and HIF-2α levels even in normoxia and the constitutive expression of a lot of hypoxia-inducible genes. After hydroxylation of their prolines, HIF-1α and HIF-2α are recognized by VHL and targeted for ubiquitination and proteasomal degradation.

Mice with germ line inactivation of VHL display an embryonic lethality due to abnormal placental vascularization (Biju et al., 2004). VHL plays an essential role not only in normal

placental development but also in the development and differentiation of many other tissues. VHL participates in the regulation of the extracellular matrix, apoptosis, control of cell senescence and transcriptional regulation. Although less studied, VHL also has been shown to participate in HIF-independent functions by binding to other molecular partners than HIFs. These include MDM2 related to p53 function, p400 related to senescence and CK2 association with AKT and NF-κB pathways among others (M. Li & Kim, 2011).

1.6.3 Role of HIF-1 in immunity

As indicated above, HIF-1 is triggered by hypoxia, inflammation or infection during normoxia and is involved in the regulation of both innate and adaptive immune responses. Diverse studies have shown that stabilization of HIF-1 can control inflammatory responses and contribute to clearance of infections. HIF-1 is an immunoregulatory transcription factor and it can promote antimicrobial activities of myeloid cells and regulate differentiation and activation of lymphocytes.

1.6.3.1 HIF-1 in macrophages and DCs.

HIF-1 in myeloid cells has not been shown to control the development and differentiation of monocytes and neutrophils, but it is important for the inflammatory responses of macrophages and their motility and aggregation (Cramer et al., 2003). During group A Streptococcus infection in mice, HIF-1 deficiency hampers the antimicrobial activity of macrophages and neutrophils without affecting phagocytosis resulting in increased bacterial titers and severe disease. HIF-1 can also stimulate NO and TNF production. The recruitment of myeloid cells and angiogenesis in tumors are enhanced by HIF-1 with detrimental consequences for the host (Palazon et al., 2014). As indicated above HIF-1 drives metabolic reprogramming of immune cells with increased glycolysis and reduced oxidative phosphorylation, which is necessary for their activation.

The upregulation of the expression of CCR7 chemokine receptor is required for the migration of DCs towards the dLN. This is a critical step required for activation of naïve antigen specific T cells, and its absence is important for maintenance of immune homeostasis. CCR7 stimulation will activate the HIF-1 transcription factor pathway and result in a metabolic shift to glycolysis in DCs. The enhanced glycolysis is required by DCs for their migration to dLN where antigen presentation to T cells, expression of costimulatory receptors and initiation of adaptive immune responses take place (J. Liu et al., 2019).

1.6.3.2 HIF-1 in T cells.

The deletion of VHL in early T cell maturation in the thymus promotes HIF-1 expression and causes an increased apoptosis of $CD4^{+}CD8^{+}$ double positive thymocytes (Biju et al., 2004), whereas the deletion of HIF-1 on early DN thymocytes instead causes no major effects on thymocyte maturation.

A growing body of evidence shows the significance of HIF-1 in the metabolism, differentiation and functions of matured T cells. Upon activation, the T cell metabolism shifts from oxidative

phosphorylation (OXPHOS) to glycolysis based on the bioenergetic demands for proliferation and differentiation. The cellular metabolism changes back to oxidative phosphorylation when the pathogen is cleared, and a small population of memory T cells survive (van der Windt & Pearce, 2012). CD4 helper T cells differentiated in vitro are more glycolytic than in-vitro-induced Treg cells which depend more on lipid oxidation and OXPHOS. Cytokines might regulate metabolic pathways which in turn affect T cell differentiation and functions. For example, IL-15 promotes cellular mitochondrial fatty acid oxidation, which contributes to CD8 T cells survival and their development into memory cells (Phan & Goldrath, 2015; van der Windt et al., 2012). HIF-1 is well known for its role in promoting glycolysis and inhibiting oxidative phosphorylation (Taylor & Scholz, 2022). Increased HIF-1 has been found to support T_H17 differentiation (Phan & Goldrath, 2015). Hypoxia or HIF-1α expression can directly induce RORγt which is required for T_H17 differentiation and production of inflammatory cytokine IL-17. Glycolysis inhibition also blocks the development of T_H17 cells. In this process, the differentiation is initiated via the cytokines IL-6 and TGF-β in a STAT3-dependent manner (E. V. Dang et al., 2011). In addition, HIF-1α has been shown to bind to forkhead box P3, which is the transcription factor for Treg cells for its proteasomal degradation thereby inhibiting Treg differentiation (Corcoran & O'Neill, 2016). Moreover, HIF-1-stimulated lactate dehydrogenase A, an enzyme required to sustain glycolysis by hampering the TCA cycle, upregulates the IFN-γ expression by T_H1 cells (Peng et al., 2016).

The loss of HIF-1 and HIF-2 in CD4 T cells compromise germinal center B cell formation, the generation of T_{FH} cells and antigen-specific antibodies after sheep blood cell immunization in mice (S. H. Cho et al., 2019). VHL in T cells is found to be essential for T_{FH} cells development and differentiation in the mice infected with LCMV. HIF-1 is also shown to be required for IFN-γ production by T cells (Cho et al).

In contrary to the data above, the percentage and numbers of T_{FH} cells defined by surface markers such as CXCR5, ICOS and PD-1 were reduced in mice lacking Vhl in mature T cells (Zhu et al., 2019). A similar effect of VHL deletion on T_{FH} cell differentiation is shown by Bonnie *et al.* using in vivo CRISPR-9 screening (B. Huang et al., 2022).

Mice deficient for VHL in T cells display improved protective CD8 T cells responses during chronic infection with LCMV and tumors (Doedens et al., 2013). Moreover, HIF-1 and HIF-2 in CD8 T cells support the differentiation of tissue-resident memory-like tumor-infiltrating lymphocytes (Liikanen et al., n.d.). HIF-1 is shown to promote the anti-tumoral functions of CD8 T cells through increasing the expression of VEGF-α. In both conditional knockout mice for HIF-1α and VEGF-α in T cells, CD8 T cell migration and anti-tumoral functions are impaired causing accelerated tumor growth (Palazon et al., 2017; Sukumar et al., 2013).

1.6.4 Role of HIF-1 in *M. tuberculosis* infection

It has long been known that human TB granuloma is hypoxic. Recent data showes that HIF-1 expression is increased in granulomas of *M. tuberculosis*-infected mice that are extremely susceptible to the *M. tuberculosis* infection (Harper et al., 2012; Osada-Oka et al., 2019).

The role of HIF-1 in myeloid cells has been studied during *M. tuberculosis* infection. Mice lacking HIF-1α in myeloid cells (*hif1a* $^{fl/fl/fl}$ *Lysm cre*) are more susceptible to *M. tuberculosis* due to the reduced levels of diverse effector molecules. HIF-1-deficient macrophages are permissive to *M. tuberculosis* infection even in presence of IFN-γ. HIF-1α expression by myeloid cells improves the control of *M. tuberculosis* both in a IFN-γ-dependent manner by enhancing the production of prostaglandins and NO in macrophages, and in an IFN-γ-independent manner which then requires CD4 T cell-derived GM-CSF for the control (Braverman et al., 2016; Van Dis et al., 2022). HIF-1 controls the metabolic shift to glycolysis by preventing the accumulation of pyruvate by the expression of lactate dehydrogenase A. In absence of HIF-1, the higher macrophage levels of pyruvate will fuel *M. tuberculosis* growth within the phagosomes (Osada-Oka et al., 2019). In addition, the molecular constitution of lipid droplets in *M. tuberculosis*-infected macrophages are regulated by HIF-1. HIF1-α and IFN-γ mediated lipid droplet formation leads to the protective eicosanoid prostaglandin E2 accumulation, rather than working as a source of nutrients for *M. tuberculosis* (Knight et al., 2018).

On the other hand, the NO/ iNOS induction has been shown to promote HIF-1α expression generating a positive feedback loop of antimicrobial function while the same time limiting excessive inflammation driven by NF-κB (Braverman & Stanley, 2017). The protective role of HIF-1α in conferring antimicrobial activity against *M. tuberculosis* is further validated in human macrophages incubated with an approved PHD inhibitor (Zenk et al., 2021).

However, the role of HIF-1 in *M. tuberculosis* infection is still under debate. Moerida *et al.* shows that it is hypoxia in human TB lesions and HIF-1α induced by *M. tuberculosis* infection which drive matrix metalloproteinase-1 expression leading to lung destruction (Belton et al., 2016). Moreover, HIF-1 in myeloid cells has been indicated not to be required for early control of *M. tuberculosis* growth but can rather prevent the immunopathological changes during chronic infection (Resende et al., 2020).

1.7 DM-TB CO-MORBIDITY

Diabetes mellitus (DM) characterized by increased levels of blood glucose over a prolonged time, is currently one of the largest groups of chronic metabolic diseases. It was estimated that for adults aged 25 years and older, the global prevalence of diabetes was around 10% (Danaei et al., 2011). In 2017, approximately, 451 million people were suffering from diabetes worldwide, and this number was expected to rise by 49% to 693 million by 2045(N. H. Cho et al., 2018). Diabetes is classified into type 1 diabetes, type 2 diabetes, gestational diabetes mellitus and rare cases of diabetes by other causes, for example pancreatic trauma and is characterized by elevated blood glucose.

Type 2 diabetes (DM) is caused by a progressive loss of insulin resistance which is also followed by an insufficient β-cell insulin secretion which frequently accounts for 90-95% of all diabetes cases (American Diabetes Association, 2019; Kanter & Bornfeldt, 2016). Methylglyoxal (MGO) can be formed as a byproduct of glycolysis, but it is detoxified by the

glyoxalase system. MGO, a highly reactive dicarbonyl compound, is stabilized not only by hyperglycemia, but also by inflammation and hypoxia as increased glycolysis and reduced enzyme activity of glyoxalase are found under these conditions (Schalkwijk & Stehouwer, 2020). Formation of advanced glycation endproducts (AGEs) is the result from MGO via a nonenzymatic glycation of proteins, lipids and DNA (Maessen et al., 2015). The AGEs as well as MGO can contribute to insulin resistance and β-cell dysfunction, which predisposes to DM, vascular complications of DM and some age-related inflammatory diseases. While MGO might have different roles in different immune cell populations like T cells and macrophages, most studies show that MGO impairs cell activation, phagocytosis and promotes cell apoptosis (X. Zhang et al., 2022).

High blood sugar can weaken a person's immune defense. Many infections are associated with DM including sepsis, TB, meningitis, endocarditis, cellulitis et al (Carey et al., 2018). DM has been reported to be a risk factor for TB for more than 40 years. TB patients with DM represent a combination that is prevalent in low- and middle-income countries. Individuals with DM have a 3-fold higher risk than those without DM to develop TB, and the TB burden attributable to DM is 15%. The failure of antibiotic treatment, TB reactivation and mortality are increased in TB patients with DM. Moreover, TB may also worsen the metabolic control in patients with DM (Ngo et al., 2021; Ugarte-Gil et al., 2019; Workneh et al., 2016). Diverse immune cells including T cells, macrophages and B cells are involved in DM with T cells and macrophages mediating the inflammatory processes observed. The immunological dysfunctions probably make DM patients more prone to *M. tuberculosis* infection (Ayelign et al., 2019). Some studies in which a functional deficiency of macrophages and alterations of T cells responses were found in TB patients with DM might explain a part of the immunological basis in TB-DM co-infection (Ronacher et al., 2015).

In LTBI patients with coincident DM, the *M. tuberculosis*-specific T_H1, T_H2 and T_H17 cell responses are diminished partially IL-10 and TGF-β dependent manner (N. P. Kumar et al., 2016). Furthermore, increased TNF-α and IL-10 are observed in human macrophages stimulated with *M. tuberculosis* under hyperglycemic conditions (Lachmandas et al., 2015). However, in active TB patients with DM, the frequencies of *M. tuberculosis*-specific single- and double-cytokine-producing T cells are elevated (Kumar Nathella & Babu, 2017). Thus, T cell responses are altered in DM-TB comorbidity.

As described above, HIF-1 is critical for cellular adaptation to oxygen deprivation and regulation of immune responses in infectious and inflammatory diseases. HIF-1 in myeloid cells has been shown as a key to control of *M. tuberculosis* infection in an IFN-γ or iNOS dependent manner (Elks et al., 2013). However, MGO induced by hyperglycemia results in proteasomal degradation of HIF-1α through interacting with heat shock protein (Hsp) 40 and Hsp70 which binds to CHIP, an E3 ubiquitin ligase. Transactivation activity of HIF-1 can also be suppressed by hyperglycemia partially mediated by MGO that modifies p300 inhibiting recruitment of HIF-1α or inhibiting the dimerization of HIF-1α there with HIF-1β (Catrina & Zheng, 2021).

The increased inflammation and bacillary loads in lungs are observed in hyperglycemic mice infected with *M. tuberculosis* (Cheekatla et al., 2016; Martens et al., 2007). The susceptibility of *M. tuberculosis* infection by hyperglycemia might be partially explained by the impaired functions of "diabetic" macrophages (Lopez-Lopez et al., 2018; Martinez et al., 2016). Besides, high glucose levels impede in vitro granuloma formation leading to a reactivation of dormant *M. tuberculosis* by mycobacterial resuscitation promoting factors (Verma et al., 2021).

Altogether, hyperglycemia in DM patients contributes to a higher susceptibility to *M. tuberculosis* infection. Diverse pathways, cellular and molecular changes involved in the increased susceptibility have been presented but the mechanisms underlying the diminished control of infection still need to be further explored.

2 RESEARCH AIMS

1. Paper I: Study the role of HIF-1 in T cells during *M. tuberculosis* infection and BCG immunization

2. Paper II: Compare recirculating and tissue resident T cell responses in lung and dLN during *M. tuberculosis* infection and mucosal and distal BCG immunization

3. Paper III: Explore whether a regulation of HIF-1 accounts for the diminished bacterial control in macrophages infected with *M. tuberculosis* treated with high-glucose or methylglyoxal (MGO)

3 MATERIALS AND METHODS

The details of materials and methods from all studies included in this thesis are shown in the three publications. In this section, I will talk on the overall features of the mouse models and on main in vivo and in vitro methods we used in this thesis.

3.1 MOUSE MODELS USED

Due to logistical and ethical considerations, experimental research in humans has been limited for mechanistic immunology research (Masopust et al., 2017). Although genetic differences exist between mice and humans, mice resemble humans in many immunological aspects and have substantial advantages for research of human immunology (Rydell-Törmänen & Johnson, 2019). Here, we apply conditional knockdown mice generated using the Cre-lox combination system, in which the Cre DNA recombinase is expressed under a cell-specific promoter. Although the target gene is flanked by LoxP sequences in all cells for recognition by the Cre enzyme, it will only be deleted specifically in Cre-expressing cells. In *Vhl*$^{fl/fl}$ *cd4 cre* mice, cre recombinase expression is driven by the T cell-specific CD4 promoter. *Hif1α*$^{fl/fl}$ *dlck cre* mice possess a conditional knockdown of *Hif1a* generated by a cre-recombinase expressed under the control of the distal promoter of the lymphocyte protein tyrosine kinase gene (dlck). Both CD4 and dlck promoters are expressed at the late stage of T cell development and are supposed to be used for impacts of specific targets on T cell function. *Vhl*$^{fl/fl}$ *cd4 cre* mice have been shown to have normal T cell development in the thymus and in *Hif1α*$^{fl/fl}$ *dlck cre* mice no effects on CD8 T cell proliferation were found (Palazon et al., 2017; Zhu et al., 2019). The mouse models we used in this thesis are shown as below:

Mouse strain	Type of modification	effect
Vhl$^{fl/fl}$ cd4 cre (*Vhl cKO*)	conditional knockdown	deficient *Vhl* in mature T cells
Hif1α$^{fl/fl}$ dlck cre (*Hif1a cKO*)	conditional knockdown	deficient *Hif1α* in mature T cells
Vhl$^{fl/fl}$ and Hif1α$^{fl/fl}$ cd4 cre (*Vhl Hif1a dcKO*)	conditional knockdown	deficient *Vhl* and *Hif1α* in mature T cells
Ly5.1	congenic mice of B6 strain	CD45.1 is expressed in hematopoietic cells except erythrocytes and platelets
Rag2$^{-/-}$	knockout	disruption of the *Rag2* gene in all cells no mature B and T cells
BKS-Lepr$^{db/db}$/JOrlRj (*Lepr$^{db/db}$*)	Mutation of the Lepr^{db} gene located in chromosome 4	Compensatory hyperplasia of the β cells in the islets of Langerhans

3.2 *M. TUBERCULOSIS* INFECTION

To mimic human TB, mice were infected with *M. tuberculosis* infection via aerosol in all the experiments (Bhaskar & Upadhyay, 2003). The dose of infection used was ca. 250 colony forming units (CFUs) and was estimated by measuring the bacterial CFUs in the lungs one day after infection. A nose-only aerosol exposure unit (In-tox Products, New Mexico, USA) was used for the infection. This method is based on the nebulization of a *M. tuberculosis* containing solution, which is inhaled by mice for 20 minutes. The advantages of an aerosol infection compared to an intravenous route are several. It is arguably the experimental model that better reproduces the route of transmission in the natural infection with *M. tuberculosis* in man. This methodology results in a lower number of infecting CFUs, a faster lung pathology, shorter survival time, and higher rate of bacterial growth in mice as compared with the intravenous infection route. Moreover, while the lung is the primary target in the aerosol and i.v. delivery routes, alveolar macrophages in the lumen are the first targets only after aerosol infection (Schwebach et al., 2002).

3.3 NAÏVE CD4 T CELL ACTIVATION IN VITRO

To better understand how HIF-1 regulates CD4 T cell responses, naïve CD44^{-} CD4 T cells were isolated from the spleens of wild type (WT) and the genetically modified mice described

above by negative selection using magnetic beads, reaching a purity of approximately 90%. Naïve CD4 T cells were stimulated with anti-CD3/CD28 in vitro as shown in figure 7. For some experiments, naïve CD4 T cells were also stimulated with superantigen staphylococcal enterotoxin B, which also binds TCR and induces T cell activation and proliferation. In other studies, CD4 T cells were stimulated with phorbol myristate acetate (PMA)/ ionomycin in order to bypass TCR signaling.

The T cell activation, cell cycle, size growth, proliferation, apoptosis, differentiation, expression of chemokine receptors and cytokine secretion were investigated in wild type (WT) and mutant CD4 T cells by flow cytometry.

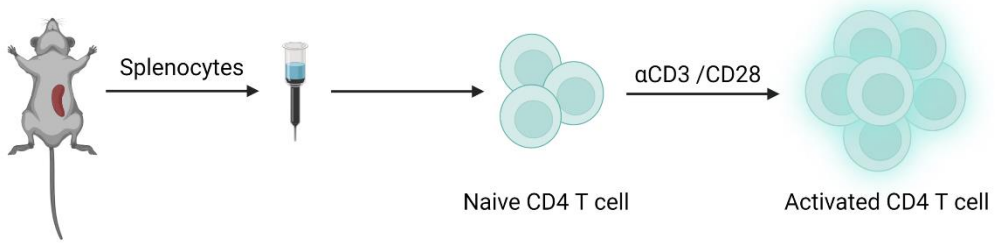

Figure 7: **Naïve CD4 T cell activation in vitro.** *Created with Biorender.com.*

3.4 INFECTION OF BMM WITH GFP-*M. TUBERCULOSIS*

To investigate the intracellular growth of *M. tuberculosis*, H37Rv carrying the green fluorescent protein (GFP)-encoding pFPV2 was used for infection of BMM. We used the transgenic fluorescent protein to quantify *M. tuberculosis* by flow cytometry, or to identify and analyze infected cells. The GFP was amenable to fixation, so infected BMM could be quantified outside a BSL3 laboratory (Mily et al., 2020).

3.5 ADOPTIVE T CELL TRANSFER IN MICE

To address whether VHL-deficiency in CD4 T cells hampers the protective capacity against *M. tuberculosis* or not, 2×10^6 naïve *Vhl cKO* or WT CD4 T cells were transferred to *Rag2*$^{-/-}$ mice 3 days after *M. tuberculosis* infection. Then the bacterial loads in lungs and spleens and T cell numbers in lungs from those infected *Rag2*$^{-/-}$ mice were evaluated4 weeks after T cell transfer. The scheme of adoptive CD4 T cell transfer used in this thesis is shown in figure 8.

Impaired proliferation in *Vhl cKO* CD4 T cells was observed both in vitro and in vivo experiments under certain stimuli. Homeostatic proliferation of T cells occurs when small numbers of T cells are adoptively transferred into immunodeficient hosts which requires contact with self-MHC molecules. To further investigate homeostatic proliferation ability of *Vhl cKO* CD4 T cells, 2×10^6 Ly5.1 (WT) and *Vhl cKO* CD4 T cells were separately or together transferred into *Rag2*$^{-/-}$ mice. The percentages and cell numbers of CD4 T cells in spleen and blood were then measured 5 weeks after cell transfer. The method used for this co-transfer study is same as the one used for *M. tuberculosis* infected *Rag2*$^{-/-}$ mice described above.

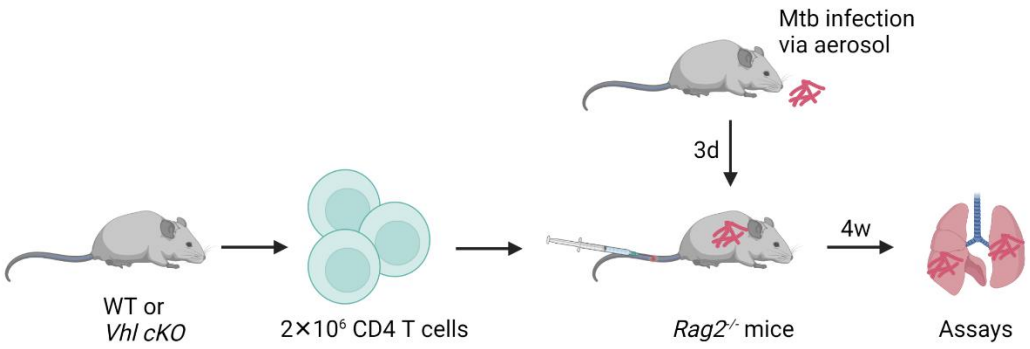

Figure 8: **Scheme of adoptive CD4 T cell transfer.** *Created with Biorender.com.*

3.6 INTRAVASCULAR STAINING

Antigen-specific T cells located in lung parenchyma mediate a better control of *M. tuberculosis* than those located in intravascular (Sakai et al., 2014). CD45 is surface maker expressed on all leukocytes, so intravascular staining of CD45.1 or CD45.2 differentiate lung parenchyma-localized from blood-borne cells (Anderson et al., 2014). In some experiments used in paper I and II, we injected mice with anti-CD45.2 i.v. 3-5 minutes before their euthanization, then CD45.2 labelled T cells were defined as blood-borne cells.

4 RESULTS AND DISCUSSION

4.1 PAPER I: HIF-1 STABILIZATION IN T CELLS HAMPERS THE CONTROL OF *MYCOBACTERIUM TUBERCULOSIS* INFECTION

Although human TB has existed for several thousand years, it remains a global public health problem by causing around 1.5 million deaths per year. BCG is the vaccine used against TB, but it provides variable protection especially in adults. As T cell responses essentially contribute to TB control by secreting IFN-γ which then activate infected macrophages to kill intracellular *M. tuberculosis*, those have been a major focus of TB immunology research. HIF-1, a transcription factor accumulated in hypoxia and T cell activation, has been recently shown to be important for CD8 T cell differentiation and effector function during virus infection and cancer (Doedens et al., 2013; Palazon et al., 2017). In macrophages, HIF-1 enhances the production of IFN-γ and NO as well as it improves *M. tuberculosis* control (Braverman et al., 2016; Braverman & Stanley, 2017). Granulomas formed in guinea pig, rat, nonhuman primate, C3HeB/FeJ mouse (good models for granuloma formation in mice) and human infected with *M. tuberculosis* are hypoxic and HIF-1 accumulation is detected in macrophages infected with *M. tuberculosis*. Thus, we hypothesize that HIF-1 in T cells plays a role in regulating T cell responses during *M. tuberculosis* infection.

To evaluate whether VHL and HIF-1 could regulate T cell responses during *M. tuberculosis* infection, we first infected *Vhl cKO*, *Hif1a cKO* and WT mice with *M. tuberculosis* via aerosol. We did not find any differences of bacterial loads in lung and spleens *Hif1a cKO* and WT mice, which was in line with similar T cell responses in the lungs. However, we detected increased *M. tuberculosis* titers in lungs and spleens from *Vhl cKO* mice at 4, 6 and 8 weeks after infection in comparison to WT mice. *Vhl cKO* mice showed a reduced weight and survival as well as reduced total and specific T cell accumulation in the lungs, especially for CD4 T cells. The *Vhl cKO* T cells from infected mice produced less IFN-γ after restimulation with *M. tuberculosis*-specific peptides. The expression of inhibitory molecules including PD-1, CTLA-4 and CD73 were augmented, effector and chemokine receptors (KLRG1, CXCR3, CX3CR1) were diminished in lung T cells from *Vhl cKO* mice after *M. tuberculosis* infection.

Unexpectedly, a higher frequency of CD8 T_{CM} ($CD44^{+}CD62L^{+}$) cells were found in *Vhl cKO* mice both before and after *M. tuberculosis* infection. These cells with a CD8 T_{CM} phenotype were at all timepoints $CD49d^{low}$ suggesting that these are virtual memory T cells, T cells that become activated in the absence of exogenous antigens, probably by recognizing endogenous structures. Virtual memory T cells have been described to rapidly respond to viruses and provide protection against infection and their presence in naïve mice is probably a consequence of homeostatic proliferation (S. Hou et al., 2021; Rolot et al., 2018).

Although WT CD4 T cells are particularly important for the protection against *M. tuberculosis* infection, *Vhl cKO* CD4 T cells were not able to confer protection against *M. tuberculosis* infection when we transferred these T cells to infected *Rag2*$^{-/-}$ mice. A similar phenotype of T cell responses was further shown in BCG immunized *Vhl cKO* mice. Collectively, mice with

Vhl-deficient T cells are more susceptible to *M. tuberculosis* infection and display reduced responses to TCR-triggering indicate a general malfunction of the *Vhl cKO* CD4 T cells.

Next, we sorted lung CD4 T cells from infected *Vhl cKO* and WT mice to compare their gene expression at the transcriptional level. The results showed upregulated transcripts involved in glycolysis, T cell exhaustion and the HIF-1 pathway, while downregulated transcripts within the OXPHOS and MYC pathway in *Vhl cKO* CD4 T cells were found. These results suggested a vital role of VHL in T cells in T cell activation, cell cycle, proliferation, and MYC-regulated cell processes. To further figure out the mechanism, we used anti-CD3/CD28 to stimulate naïve *Vhl cKO* CD4 T cells in vitro to study their response. Absence of VHL in T cells caused cell cycle arrest by freezing the cells in G2 and a decreased protein (but not mRNA) expression of MYC and its downstream target CD71. In line with MYC's importance for cell size growth, apoptosis, proliferation and cell metabolism, we observed a diminished cell size growth, proliferation, activation levels defined by CD44, CD69 and CD25 in *Vhl cKO* CD4 T cells, as well as reduced IL-2 titers the supernatants of *Vhl cKO* CD4 T cells after activation with anti-CD3/CD28. IL-2 is required for the clonal expansion of WT T cells. However, the addition of IL-2 did not recover the proliferation of *Vhl cKO* CD4 T cells. The IL-2 receptor α-chain, CD25 was induced in activated *Vhl cKO* CD4 T cell responses albeit at lower levels than in WT controls. Together the diminished production to IL-2 partially accounted for the impeded responses of the *Vhl cKO* CD4 T cells.

MYC has been shown to synergize with HIF-1 in programming T cell metabolism at various stages of T cell activation, proliferation and differentiation (Gnanaprakasam et al., 2017). However, other studies finds that HIF-1 on MYC can have antagonistic effects. As indicated in the introduction section, HIF-1 can negatively regulate MYC activity by activating MXI1, a molecule that binds to MAX, the partner required for MYC for its transcriptional function. MXI1 is thereby a competitive inhibitor of MYC function. HIF-1α could also promote MYC degradation by a still not well understood mechanism in a MXI1 independent manner (H. Zhang et al., 2007). Moreover, HIF-1 may perturb the cell cycle driven by MYC through displacing MYC from its DNA binding site and derepressing p21 (*Cdnk1a*), a cell cycle inhibitor (Koshiji et al., 2004). Similarly, higher RNA expression of *Mxi1*, transcripts of cell cycle inhibitors such as *Cdnk1a* and decreased CD71 expression, a downstream target of MYC, were displayed in activated *Vhl cKO* CD4 T cells in comparison with the control. This suggests that HIF-1α stabilization may repress MYC by displacing it from *Cdnk1a* and may upregulate MXI1 suppressing the transcription activity of MYC. Besides, MYC expression in activated *Vhl cKO* CD4 T cells was reduced as detected by western blot indicating a proteasome-mediated degradation of MYC by HIF-1α. The different pathways of MYC repression by HIF-1α are summarized in figure 9 (C. V. Dang et al., 2008; L. E. Huang, 2008).

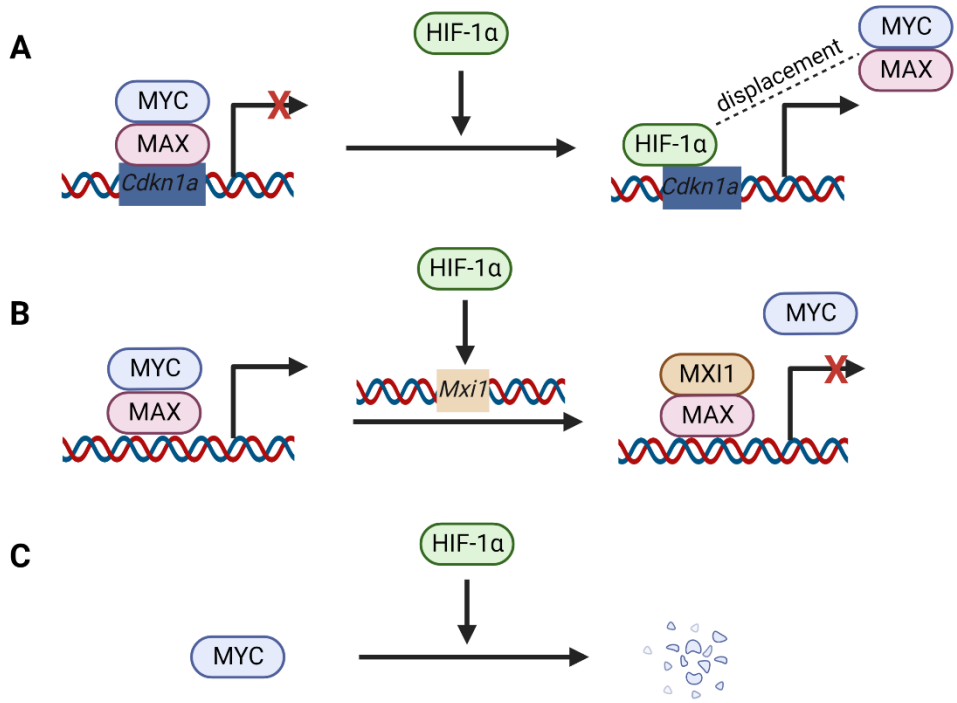

Figure 9: **Repression of MYC by HIF-1α.** *Created with Biorender.com.* A: Displacement of MYC by HIF-1α, B: HIF-1α upregulates MXI1 which then competes with MYC for MAX binding, C: MYC degradation

T cell activation and proliferation were also found impaired in *Vhl cKO* CD4 T cells stimulated with Staphylococcal enterotoxin B superantigen. Instead, PMA / ionomycin stimulated *Vhl cKO* CD4 T cells showed similar CD44, CD69 and CD25 expression with WT CD4 T cells, suggesting that the impaired T cell responses in *Vhl cKO* CD4 T cells might be mediated through TCR signaling. Then we looked at the early events after TCR activation and discovered that expression of phosphor-pS6 and erk-2 phosphorylation (unpublished data) also were reduced in *Vhl cKO* CD4 T cells after TCR activation. When stimulated with PMA/ ionomycin, bypassing TCR signaling levels of phosphor -pS6 and IFN-γ production in WT and *Vhl cKO* CD4 T cells were similar. This strongly suggests that HIF-1 stabilization affects the TCR-proximal signaling. The homeostatic proliferation of T cells in lymphopenic environments has been shown to require contact between TCR complex and self-MHC molecules. Homeostatic proliferation in mice inoculated with *Vhl cKO* CD4 T cells was also strikingly reduced.

In contrast to the results we obtained, VHL-deficient CD8 T cells have been demonstrated to increase the control of chronic virus infections and tumors with high HIF-1 levels accounting for those effects (Doedens et al., 2013; Liikanen et al., n.d.). In line, CD8 T cells deficient in HIF-1 show a compromised migration, differentiation, effector phenotype and leads to tumor progression (Palazon et al., 2017).

In CD4 T cells, it has been shown that HIF-1 promotes T_H1, T_H17 and T_{FH} cell differentiation but restrains Treg cell differentiation (Cho et al., 2019; Clever et al., 2016; Dang et al., 2011).

Contrary to these findings, HIF-1 impedes the calcium flux activation and IFN-γ production after TCR signaling in CD4 and CD8 T cells from other studies (Lukashev et al., 2006; Neumann et al., 2005). The cell cycle and the proliferation in many cell types including lymphocytes is inhibited during hypoxia in a HIF-1 dependent fashion (Hubbi et al., 2013; Hubbi & Semenza, 2015). In addition, a reduced IFN-γ secretion, proliferation and increased apoptosis of T cells activated via TCR under hypoxic conditions has been described (Caldwell et al., 2001; Larbi et al., 2010). Others find that VHL in T cells promotes T cell differentiation including T_H17 and T_{FH} cell differentiation, which is consistent with our findings (Chitrakar et al., 2020; Zhu et al., 2019).

Both hydroxylated HIF-1α and HIF-2α can be targeted by VHL for proteasomal degradation. Although HIF-1 and HIF-2 have similar functions in angiogenesis, they have distinct roles in other cellular processes. As mentioned above, HIF-1 plays an important role in glycolysis, CD8 T cell effector functions, antimicrobial activity, T cell differentiation and survival, while HIF-2 has been reported as a key regulator of tumor growth, Treg cell function, and cytotoxicity of CD8 T cells (Hsu et al., 2020; Veliça et al., 2021). Although VHL performs some of its functions in a HIF-independent way, most functions that were studied depend on a regulation of HIF by VHL.

To address whether the constitutive HIF-1 expression drives the enhanced susceptibility to *M. tuberculosis* infection of *VHL cKO* mice, we crossed *VHL cKO* with *Hif1a cKO* mice and generated *Vhl Hif1a dcKO* mice. *Vhl Hif1a dcKO* mice displayed similar numbers of *M. tuberculosis* in the lungs and spleens 4 and 7 weeks after *M. tuberculosis* infection compared to WT mice. Frequencies and cell numbers of CD4, CD8 and *M. tuberculosis*-specific T cells in lungs from *Vhl Hif1a dcKO* mice were comparable to WT mice. Further, *Vhl Hif1a dcKO* CD4 T cells behaved alike the WT CD4 T cells when stimulated in vitro. In addition, activation levels, cell growth, cell cycle, proliferation and transcriptome profiles of *Vhl Hif1a dcKO* and WT CD4 T cells were similar. We did not study the effects of HIF-2 on T cells, but the absence of HIF-1 in *VHL cKO* CD4 T cells completely restored T cell responses both in vitro and in vivo.

Overall, our data indicates that HIF-1 impairs the activation and differentiation of CD4 T cells resulting in an increased susceptibility to infection with *M. tuberculosis* and in impaired protective responses generated by BCG immunization (figure 10). As discussed above, HIF-1 in T cells may have different roles resulting in disparate consequences in response to diverse pathogens. Our findings explore different aspects of HIF-1 in modulating T cells responses and show for the first time that the stabilization of HIF-1 in T cells impairs the control of *M. tuberculosis* infection. We speculate that hypoxic environment in TB granulomas formed in human may contribute to the impaired CD4 T cell responses and poor protection against *M. tuberculosis* infection.

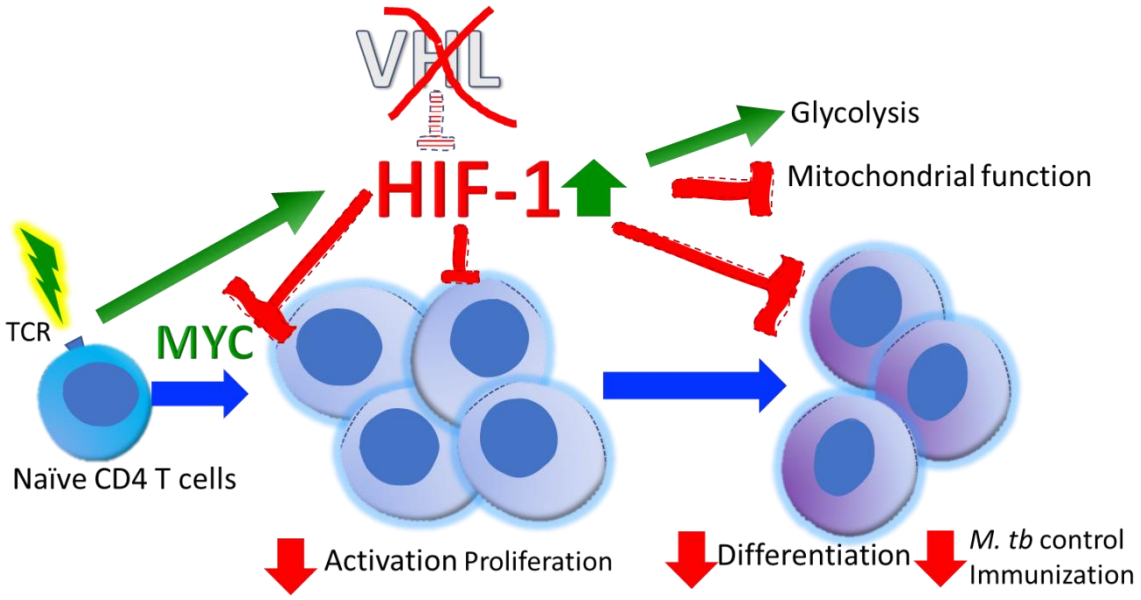

Figure 10: **HIF-1 stabilization in T cells hampers the control of infection with *M. tuberculosis* by impairing T cell activation**

4.2 PAPER II: MYCOBACTERIA-SPECIFIC T CELLS ARE GENERATED IN THE LUNG DURING MUCOSAL BCG IMMUNIZATION OR INFECTION WITH *MYCOBACTERIUM TUBERCULOSIS*

Diverse vaccination strategies of BCG are studied to improve protection efficiency against *M. tuberculosis* infection. T_{RM} generated in lung airways via mucosal BCG immunization mediate better protective T cell responses against TB compared to that of intradermal BCG immunization (Perdomo et al., 2016). *M. tuberculosis*-specific T_{RM} formed in lung parenchyma conferre potent protection against *M. tuberculosis* infection in mice by adoptive transfer (Sakai et al., 2014). However, the formation and maintenance of mycobacteria-specific T_{RM} during BCG immunization via intratracheal (i.t.) and subcutaneous (s.c.) and *M. tuberculosis* infection is not clear.

For this purpose, we infected mice with *M. tuberculosis* via the aerosol route and evaluated the presence of specific T cells in the lungs and the mediastinal dLN. Using tetramers, we measured the accumulation of TB10.4-specific CD8 T cells and of Ag85B and ESAT-6-specific CD4 T cells at several time points after infection. While specific T cells could be measured in the lung at 20 dpi, we were unable to detect *M. tuberculosis* specific T cells in the dLN. A high percentage of activated T cells were tetramer positive in the lung confirming that some of the applied tetramers presented highly immunodominant mycobacterial peptides. The frequency of $CD44^+$ CD4 and CD8 T cells was much higher in the lung than those in dLN. Consistent with this observation, IFN-γ secretion after restimulation with *M. tuberculosis*-specific peptides by CD4 and CD8 T cells in lung of *M. tuberculosis*-infected mice was higher than that in the dLN. Moreover, responses to PPD, that consists of a suspension of several mycobacterial proteins, followed the same pattern indicating that a skewed specificity of T cells in different sites does not explain the observed lower T cell responses in the dLN. Altogether our data

indicates that mycobacteria-specific T cells accumulate in the lung in the absence of a detectable presence of specific T cells in the dLN during *M. tuberculosis* infection.

Our data suggested that antigen-specific T cells in the lung of infected mice could be tissue resident. To evaluate this possibility, the presence CD4 T_{RM} ($CD69^+$ $CD11a^+$), CD8 T_{RM} ($CD69^+$ $CD103^+$) in the lungs was determined. Of those, PD-1^+ T cells have been described to have a high proliferation ability and $KLRG1^+$ T cells have been described as terminally differentiated effector cells. The accumulated mycobacteria-specific lung T_{RM} showed a similar pattern to mycobacteria-specific T cells, and they are located within the parenchyma. Mycobacteria-specific PD-1^+ T cells rather than $KLRG1^+$ T cells are generated around 2 weeks after infection with increasing numbers for some days and maintained elevated at late timepoints. Moreover, *M. tuberculosis*-specific lung T cells highly express Ki67, suggesting that these specific T cells proliferate in the lung during *M. tuberculosis* infection.

We also compared the T cell responses in the lung and dLN of mice after BCG immunization via i.t. and s.c. routes. Mice immunized i.t. generated higher frequencies of Ag85B -specific CD4 T cells and TB10.4^- specific CD8 T cells in the lungs than those in the mice immunized s.c. In line with differences of T cell responses in lung and dLN in *M. tuberculosis* infected mice, nearly no mycobacterial specific T cells accumulated in dLN in mice after immunization via both i.t and s.c. Lungs from i.t. immunized mice contained mycobacteria-specific CD4 and CD8 T_{RM} already at 1-2 weeks after immunization, whereas s.c. immunization failed to induce these cells. The i.t. immunized mice developed more PD-1^+ CD8 T cells in the lung. Instead, $KLRG1^+$ CD8 T cells were detected in lungs from s.c. immunized mice. The formation of lung T_{RM} by i.t. booster was further confirmed by treatment of immunized mice with fingolimod, which can sequester the lymphocytes in the lymph nodes resulting in lymphopenia by causing the internalization of the S1P receptors. Thus, lung T cells from mice treated with fingolimod are resident and are maintained in the absence of dLN recirculation.

Mice treated with subcutaneous BCG immunization have higher bacterial loads in the lung after *M. tuberculosis* challenge than those treated with mucosal BCG immunization, which has been demonstrated to induce a dose-dependent control (Aguilo et al., 2014; Giri et al., 2006). The antigen-specific T_{RM} have been also shown to express PD-1 in the lung parenchyma that may contribute to the enhanced protection against due to superior proliferative (memory) capacity (Bull et al., 2019).

Our data reveals that antigen specific T cells accumulate, proliferate predominantly in the lung instead of dLN after infection with *M. tuberculosis* via aerosol and immunization with BCG via the mucosal route. Moreover, we speculate that protective T cells can be primed in the lung rather than in the dLN during *M. tuberculosis* infection and i.t. BCG immunization. The lymph nodes are the site for naïve T cells encountering DCs presenting cognate antigens and co-stimulatory molecules after migration from distal infected organs via the lymphatic circulation (Bousso, 2008; Klonowski et al., 2006). Then the adaptive immune response is initiated and the clonal expanded and activated T cells migrate to the site of infection.

However, in mice, which lack secondary lymphoid organs (SLO) due to a genetic deletion of the lymphotoxin gene, influenza-specific CD8 T cells are found in the lung and virus is cleared even if clearance was delayed. SLO-deficient mice are also used during infection with *M. tuberculosis* and the results demonstrated those mice infected developed antigen-specific and memory T cells and controlled the infection eventually (T. A. Day et al., 2010). Induced bronchus-associated lymphoid tissue (iBALT) are ectopic tertiary lymphoid organs formed in the lungs of these infected mice and have been shown to provide a microenvironment supporting T cell proliferation (Moyron-Quiroz et al., 2004). In addition, T cell priming and the generation of antigen-specific cells are identified within iBALT induced during virus infection (Halle et al., 2009). Memory CD8 T cells could be formed in these $SLO^{-/-}$ mice that could respond to antigen, expand locally and clear the virus after the challenge (Moyron-Quiroz et al., 2006). In line with conventional lymphoid tissues, lymphocytes can drive stromal cells to become follicular dendritic cells and fibroblastic reticular cells which could survive for a long time independent of inflammation in the iBALTs. Using spatial transcriptomics, our group observe the accumulation of B cell and T cells in iBALT-like structures in *M. tuberculosis* infected mice with B cell clusters, and adhesion molecules and chemokines resembling lymphoid structures (Carow et al., 2019). Others find that iBALT-like structures formed in lungs from *M. tuberculosis* infected mice and non-human primates associated to the improved vaccine-induced protection against the infection (Dunlap et al., 2020).

In summary, we have demonstrated that mycobacteria-specific T_{RM} are generated and maintained in the lung parenchyma of mice after aerosol infection with *M. tuberculosis* or a i.t. mucosal immunization with BCG. The T_{RM} are maintained in the lung parenchyma independently of the recirculation from dLN and proliferate in situ. Mucosal but not subcutaneous BCG immunization resultes in the accumulation of parenchymal T_{RM} with high expression of PD-1 and low KLRG1 levels.

4.3 PAPER III: HIGH GLUCOSE AND CARBONYL STRESS IMPAIR HIF-1-REGULATED RESPONSES AND THE CONTROL OF *MYCOBACTERIUM TUBERCULOSIS* IN MACROPHAGES

The risk of developing TB is increased in people living with diabetes (DM) and the underlying immune mechanism behind is not clear yet. Activated macrophages are key for killing *M. tuberculosis* and, among other factors, HIF-1 influences the response of macrophages to *M. tuberculosis* infection (Braverman et al., 2016; Braverman & Stanley, 2017). Hyperglycemia, a hallmark of DM, suppresses HIF-1 function that leads to issues of tissue repair in DM patients (Botusan et al., 2008, 2008). We speculate that HIF-1 in macrophages is involved in the underlying mechanism of DM-TB comorbidity.

We first measured HIF-1 expression and its downstream targets in BCG and in *M. tuberculosis* infected bone marrow-derived macrophages (BMM). The expression of HIF-1α, and the levels of HIF-1-target transcripts such as *vegfa*, *pdk1* and *ldha* and others were higher in mycobacteria-infected BMM than in uninfected controls. RNA levels of *il1b* and *inos* and nitrite concentration, which are important factors for antimicrobial activities of macrophages,

were increased in mycobacteria-infected BMM. Further, the levels HIF-1-downstream targets were increased in lungs from *M. tuberculosis* infected mice. As indicated in the background, HIF-1 is induced not only under hypoxia condition but also by infection and inflammation in an oxygen-independent manner. Inflammation stimuli for example LPS increases levels of HIF-1 in macrophages (Blouin et al., 2004; Stothers et al., 2018). Thus, our results further confirme this by showing increased HIF-1 expression in mycobacteria-infected BMM.

Deferoxamine (DFO) treatment can mimic hypoxia condition as it stabilizes HIF-1 by abolishing PHDs hydroxylation (An et al., 1998; Chan et al., 2002). We found that HIF-1 responses and protein levels were elevated after DFO treatment in uninfected BMM. DFO further increased HIF-1 expression, and HIF-1-regulated transcripts in mycobacteria-infected BMM. We then studied the effects of DFO on *M. tuberculosis* growth in BMM in vitro and during the infection of mice in vivo. The addition of DFO reduced the *M. tuberculosis* levels in *M. tuberculosis*-infected BMM and moderately diminished the bacterial loads in the lungs from *M. tuberculosis*-infected mice. DFO has previously been used as an adjutant together with antibiotics treatment in mycobacteria-infected macrophages and improved the efficiency of the treatment (Cahill et al., 2021; Gokarn & Pal, 2017).

Iron is a cofactor of the PHDs required to target HIFs for degradation, and DFO acts as an iron chelator. However, iron is key for the macrophage functions as well as for the growth of *M. tuberculosis*, a bacterium that has strategies to hijack iron from the host. Therefore, we cannot exclude the possibility of DFO partially improving *M. tuberculosis* control as an iron chelator in our study is due to regulation of other pathways than HIF-1. On the other hand, we observed increased HIF-1 regulated responses in DFO-treated uninfected BMM indicating a robust stabilization of HIF-1 by DFO. DFO has been reported to activate HIF-1 and *iNOS* reporters of a murine macrophage cell line (Melillo et al., 1997). *M. tuberculosis* or LPS-stimulated human macrophages exhibite augmented glycolysis and IL-1β production after DFO treatment (Phelan et al., 2020). The effect of DFO on improving *M. tuberculosis* growth in lungs from *M. tuberculosis*-infected mice was moderate. This might suggest that HIF-1 could be a potential target for adjunctive therapy in TB patients. Besides, DFO treatment in vivo causes stabilized HIF-1 in multiple cell types which may have different outcomes. For instance, HIF-1 in macrophages supports their bactericidal function while it suppresses the responses of T cells (McGettrick & O'Neill, 2020; Palazon et al., 2014). Moreover, as discussed above, we have demonstrated that HIF-1 stabilization in mice deficient for VHL in T cells plays a detrimental role in T cell activation and protection against the infection with *M. tuberculosis*.

As indicated in the background, MGO, a byproduct of glycolysis, is formed during DM and its levels associate with insulin resistance and glucose levels (Ramachandra Bhat et al., 2019). We hypothesize that MGO could be involved in the reduction of HIF-1 responses in DM and thereby play a role in the increased susceptibility of DM individuals to TB. For this purpose, the effect of the addition of MGO in mycobacteria-infected BMM was then investigated. We found that MGO hampered HIF-1-regulated responses, inflammatory transcripts and nitrite accumulation in cell culture supernatants. Macrophages treated with MGO showed higher

intracellular growth of *M. tuberculosis*. Even though HIF-1 expression was not altered by MGO treatment, the transcriptional activity of HIF-1 was reduced, suggesting that MGO decreased HIF-1 controlled responses by repressing HIF-1 activity. Consistent with our results, hyperglycemia has been shown to hamper the transcriptional activity of HIF-1 via abrogating HIF-1α/ p300 association (Catrina et al., 2004; Thangarajah et al., 2010). In line, BCG-infected BMM exhibited a lower expression of HIF-1 regulated transcripts when BMM were exposed to high glucose levels and high glucose levels also decreased the control of *M. tuberculosis* in BMM.

Infecting leptin receptor-deficient $Lepr^{db/db}$ obese mice with *M. tuberculosis*, we found increased bacterial loads in their lungs compared to WT mice in parallel with higher glucose and HbA1c levels in their blood before and after infection. Lower HIF-1 regulated transcripts associated with lower metabolic and effector molecules were observed in lungs from infected $Lepr^{db/db}$ mice.

Taken together, we find that the treatment with DFO abrogated the effects of MGO and high glucose on HIF-1 regulated responses in mycobacteria-infected BMM. In line with this, diminished HIF-1 hydroxylation by DFO could restore HIF-1 functions that were suppressed by hyperglycemia and improved wound healing in DM (Botusan et al., 2008). Other HIF-1 stabilizers ameliorated metabolic disorders and DM complications in mice models (Lemos et al., 2011; Sugahara et al., 2020). DFO administration induced both HIF-1 and HIF-2 stabilization, so we cannot exclude a possibility of HIF-2 participation in the responses of DFO-treated BMM. On the other hand, HIF-1 deficient BMM showed reduced transcripts of metabolic and immune molecules after *M. tuberculosis* infection suggesting an indispensable role of HIF-1 in modulating the bactericidal activity of BMM. The main findings of paper III are summarized in figure 11.

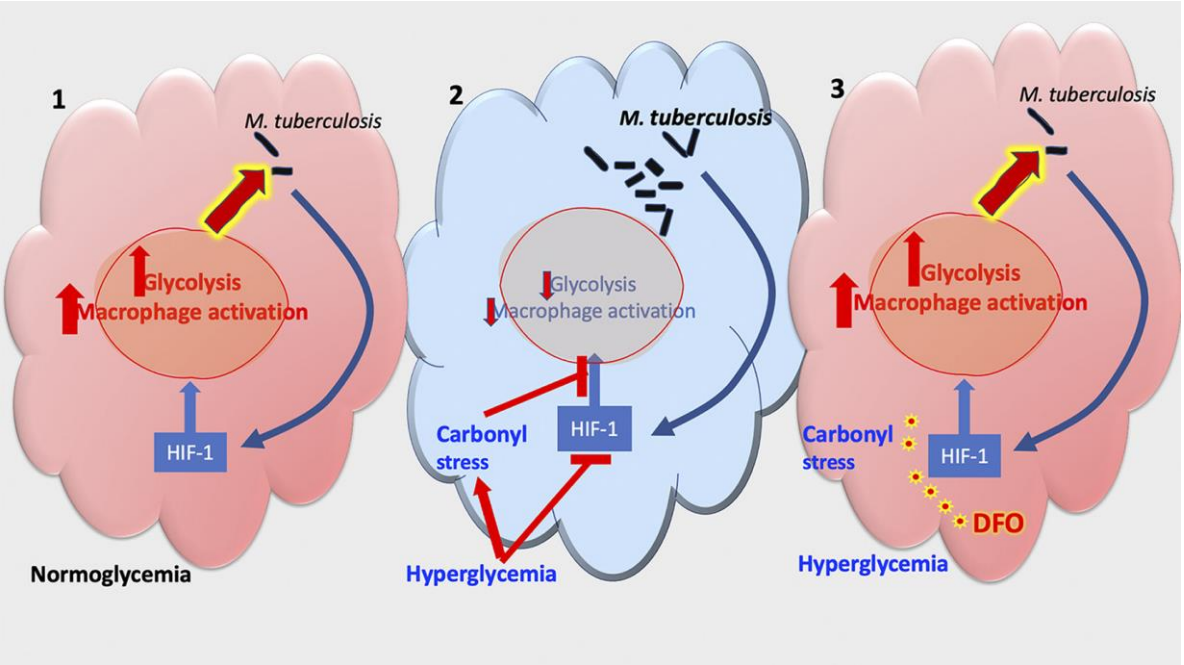

Figure 11: **Summary**. (1) *M. tuberculosis* infection induces the expression of HIF-1-regulated immunometabolic genes in BMM. These genes confer protection against *M. tuberculosis* infection. (2) During DM, high glucose environment and carbonyl molecules hinder HIF-1-regulated responses and thereby impede the control of intracellular *M. tuberculosis* in BMM. (3) Treatment with the HIF-1 stabilizer deferoxamine (DFO) increases HIF-1-regulated responses in *M. tuberculosis*-infected BMMs and in the lungs of *M. tuberculosis*-infected mice. As a consequence, DFO treatment restricts the *M. tuberculosis* growth in BMM in presence and absence of IFN-γ. Treatment with DFO also rescues HIF-1-regulated immunometabolic responses and enhances *M. tuberculosis* control in MGO and high glucose-treated BMM.

5 CONCLUSIONS AND POINTS OF PERSPECTIVE

In summary, we discussed the generation of T cell responses during *M. tuberculosis* infection and BCG vaccination, the role of HIF-1 in T cells and macrophages during *M. tuberculosis* infection alone or during comorbidity with DM in this thesis. The main findings are as shown below:

1. Stabilized HIF-1 in T cells hinders T cell activation, proliferation and differentiation
2. Mice with HIF-1 stabilization in T cells are more susceptible to *M. tuberculosis* infection

3. Mycobacteria-specific T cells are generated in the lung rather than in the dLN during *M. tuberculosis* infection or mucosal BCG immunization
4. Mucosal BCG immunization but not subcutaneous immunization induces a mycobacteria-specific T_{RM} profile in lung parenchyma

5. DM might increase risk for *M. tuberculosis* infection by repression of HIF-1
6. HIF-1 stabilization in high glucose- or MGO-treated BMM by DFO restores HIF-1 regulated responses and the control of *M. tuberculosis* growth by BMM

As the currently used typical intradermal BCG vaccination fails to generate a high vaccination efficacy against *M. tuberculosis* infection in adults, this might be attributed to insufficient accumulation of antigen-specific T cells in lung and could possibly be improved by mucosal administration.

Since HIF-1 plays different roles in T cells and macrophages during *M. tuberculosis* infection, the infection outcome of HIF-1 stabilization in granuloma which is characterized by hypoxic environment is still inconclusive. Therefore, the application of HIF-1 stabilizers in different stages of infection should be further studied.

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