Tuberculosis & type 2 diabetes

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CHAPTER

1

General Introduction

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Mycobacterium tuberculosis: an ancient but agile microbe

Tuberculosis (TB) is an infectious disease of the lungs which is caused by infection with *Mycobacterium tuberculosis* (*Mtb*). Even though the first evidence of human TB infection can be traced back many millennia to the Neolithic era (~9,000 years ago)[1], it remains a major and important threat to global health to date. In 2017, an estimated 10 million people fell ill with TB and 1.6 million died as a result of the disease, making TB the leading cause of death by a single infectious agent worldwide[2]. TB ranks among the global top 10 causes of overall mortality. In total, 87% of the global TB burden is accounted for by 30 countries, of which India (27%), China (9%) and Indonesia (8%) encompass the top three of estimated new cases. The recommended TB treatment regimen consists of four first-line antibiotics (isoniazid, rifampicin, ethambutol and pyrazinamide) for a period of 6 months. However, multidrug-resistant TB (MDR-TB) and extensively drug-resistant TB (XDR-TB) comprise a major global health challenge due to a global rise in resistance to first-line drugs. Although treatment success rates of drug-susceptible TB are approximately 85% at an estimated cost of 40 US$ per person, they drop to 55% for treatment of MDR-TB using second-line drugs with higher toxicity at significantly increased costs (>1000 US$ per person). Furthermore, while vaccination with Bacille Calmette-Guérin (BCG), a live-attenuated *Mycobacterium bovis* vaccine which has been administered to humans since 1921, shows limited efficacy in preventing disease in children, a vaccine which induces adequate protection in adolescents and adults is still lacking[3]. Recently, the efficacy of two candidate subunit vaccines (M72/AS01$_E$ and H4:IC31) for prevention of TB disease resp. infection in adults resp. adolescents was examined in phase 2 trials. Administration of M72/AS01$_E$ to *Mtb*-infected adults resulted in 54.0% protection against TB[4]. Although H4:IC31 vaccination did not lead to prevention of primary QuantiFERON-TB Gold In-tube assay (QFT) conversion in adolescents from a high TB risk setting, it did show a trend towards reduced sustained QFT conversion for 3-6 months after initial measurement[5]. Importantly, a similar but significant effect was reported for revaccination with BCG in this trial, warranting further studies into the benefits of BCG revaccination for prevention of sustained QFT conversion[5].

TB is spread through inhalation of mycobacteria-containing aerosols produced by sneezing or coughing, leading to *Mtb* infection of resident alveolar macrophages. The immune response to *Mtb* is characterized by formation of granulomas, complex immunological structures which shield the host from bacterial dissemination but simultaneously provide a niche for *Mtb* persistence[6]. First, additional mononuclear cells are recruited to the site of primary infection from neighboring blood vessels, which subsequently become infected by the expanding mycobacterial population and together form the early granuloma. After an initial delay (14-21 days) in the onset of adaptive immune responses, B and T lymphocytes are recruited to the granuloma, eventually leading to arrested *Mtb* growth but not to complete bacterial elimination. During this time of immunological deadlock the granuloma matures, which involves differentiation of

macrophages into lipid-loaded foamy, epithelioid and multinucleated giant cells and the formation of a fibrous cuff around the macrophage-rich layer. Whereas the early granuloma was adequately vascularized, *Mtb* now has to adapt to conditions of local hypoxia and nutrient-scarcity and consequently enters a state of dormancy, ensuing in a clinically asymptomatic period of latent TB infection (LTBI). It is estimated that approximately one quarter of the world's population is latently infected with *Mtb*[7], 5-10% of which will reactivate and develop active disease during their life-time. The factors which determine granuloma outcome and TB reactivation are not clearly defined, but immune components appear key players. Induction of immune mediators on opposite sides of the inflammation spectrum result in a delicate immune balance. Pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ) are major contributors to TB immunity. Treatment with TNF-α neutralizing antibodies led to reactivation of TB in humans[8] and cynomolgus macaques[9]. Mutations in the IFN-γ gene[10], the IFN-γ receptor[11] or the interleukin (IL)-12/-23 axis[12], which governs IFN-γ production[13], impair anti-mycobacterial immunity. However, high levels of pro-inflammatory factors can also lead to extensive tissue damage, resulting in liquefying caseous necrosis, pulmonary cavitation and ultimately bacterial dissemination[14].

As stated above, *Mtb* primarily infects macrophages, phagocytic cells of the myeloid lineage which play pivotal roles in immunity by direct microbe qal killing and presenting antigen to naïve T cells to induce adaptive responses. The primary route of killing or controlling intracellular bacteria is through the phagolysosomal pathway (**Figure 1**). After receptor-mediated uptake, bacteria are contained in compartments called phagosomes which rapidly mature by fusing with lysosomes, leading to vesicle acidification and the acquisition of antimicrobial peptides and hydrolases. To circumvent its eradication, *Mtb* inhibits phagosome maturation by manipulating the activity of GTP-binding Rab proteins which coordinate intracellular vesicle trafficking[15,16], through targeting Vacuolar-type H^+-ATPase for degradation to prevent phagosomal acidification[17], and through blocking the function of the NADPH oxidase complex[18], an important phagosomal component which produces antibacterial superoxide. Furthermore, *Mtb* induces perforation of the phagosome through expression of the ESX-1 secretion system[19, 20], an important virulence factor which is absent in the non-virulent BCG strain. ESX-1 effector proteins ESAT6 and CFP10 have been shown to induce phagosomal membrane damage in macrophages[19-22], simultaneously halting vesicle maturation and providing access for *Mtb* to release important effectors in the cytosol. An unwanted consequence of phagosomal escape from the perspective of *Mtb* is the induction of autophagy which serves as an auxiliary host pathway for bacterial clearance, although its importance for TB outcome is still a topic of debate[23]. Multiple autophagy pathways have been implicated in *Mtb* killing, including the detection of mycobacterial DNA by the STING-cGAS cytosolic surveillance pathway[24,25], the recruitment of host galectins to damaged phagosomes[26, 27] and ubiquitination of escaped mycobacteria by ubiquitin ligases such

as Parkin and Smurf1[28, 29], among others[30]. These pathways culminate in the recruitment of autophagy adaptors such as NDP52 and p62, which consecutively bind to membrane protein LC3 leading to the formation of degradative double membrane vesicles named autophagosomes. Again, *Mtb* has developed mechanisms to actively inhibit these clearance mechanisms, for instance by inducing expression of miR-33[31], miR-155[32] and IL-10[33], all negative regulators of autophagy. Collectively, these strategies of *Mtb* to survive the hostile intracellular environment of the macrophage are major contributors to its pathogenic success.

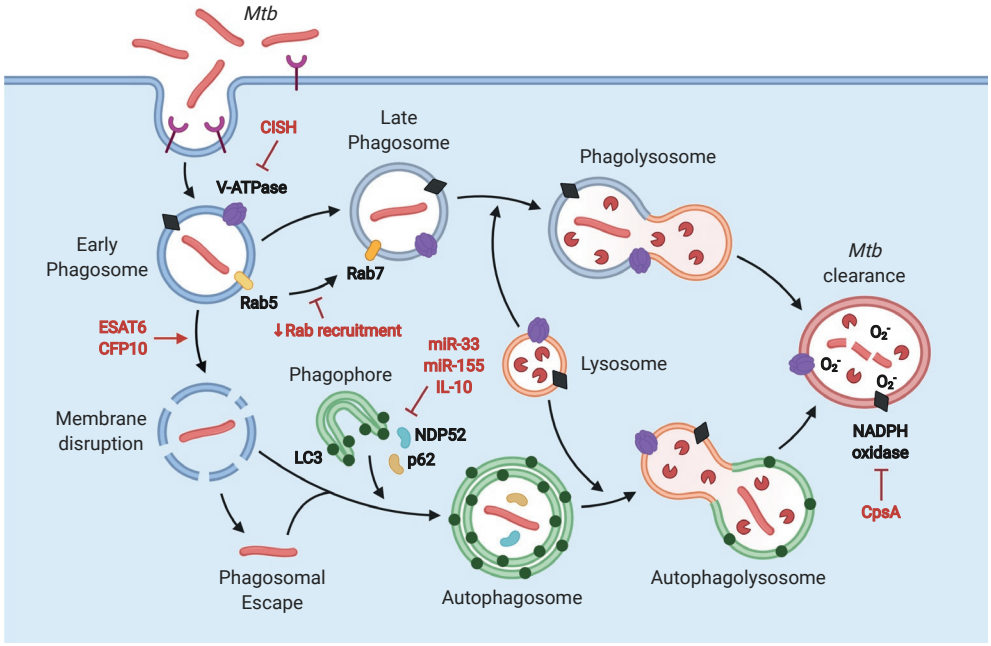

Figure 1: Overview of macrophage central energy metabolism.

After receptor-mediated uptake, *Mtb*-containing phagosomes mature by fusing with lysosomes, leading to vesicle acidification and ultimately mycobacterial eradication. *Mtb* can interfere phagosome maturation at multiple steps, including: 1) inhibition of Rab activity and recruitment, 2) induction of cytokine-inducible SH2-containing protein (CISH) to degrade Vacuolar-type H+-ATPase (V-ATPase), 3) inhibition of NADPH oxidase complex by Cold shock protein A (CspA), 4) phagosomal membrane disruption by ESX-1 effector proteins ESAT6 and CFP10 leading to *Mtb* escape. Autophagy can serve as an auxiliary pathway for *Mtb* killing, leading to autophagosome formation after binding of autophagy adaptors such as p62 and NDP52 to LC3. *Mtb* can negatively affect autophagy through induction of host microRNAs (miR-33 and miR-155) and IL-10.

Macrophage biology and immunometabolism during *Mtb* infection

While their importance in *Mtb* pathogenesis is undisputed, referring to macrophages as a single uniform cell type would be inaccurate. Macrophages exist in many different forms and functional states, and their significant plasticity has been the topic of many studies over the last decades. Initially, a dichotomic model of macrophage activation was proposed, spanning from pro-inflammatory 'classically activated' [34] to anti-inflammatory 'alternatively activated' [35] macrophages, which were later respectively labeled M1 and M2 macrophages, the myeloid equivalent of the classical Th1/Th2 paradigm[36]. M1 polarization can be induced by Th1 cytokines (IFN-γ and TNF-α) and LPS, while M2 macrophages can be generated by Th2 cytokines IL-4 and IL-13. Alternatively, macrophage differentiation of monocytes using either M-CSF or GM-CSF also results in diametrically opposed functional phenotypes (termed Mφ1 and Mφ2)[37,38]. In analogy to the over simplistic Th1/Th2 paradigm (following the discovery of Th17, Th22, Tfh and other T cell subsets), the initial M1/M2 dichotomy soon became challenged, and variations in differentiating stimuli were shown to result in phenotypically distinct macrophage subsets not conforming to classical M1/M2[39]. An extensive transcriptomics study on macrophage polarization under 29 differentiating conditions revealed a spectrum of macrophage activation which was far beyond what could be explained by the M1/M2 model, including distinct gene expression modules involved in granulomatous inflammation after stimulation with TNF-α, prostaglandin E2 and Pam3Cys[40]. Moreover, it has become clear that many tissue-resident macrophage populations consist of self-renewing cells derived from embryonic precursors, such as Kupffer (liver) and Langerhans cells (skin), which do not rely on circulating blood monocytes during steady-state[41], suggesting different lineages within the macrophage "compartment". Combined, these findings have led to the rejection of the original binary view of macrophage polarization, and have given way to multidimensional models which take into account the effects of macrophage ontogeny and local tissue microenvironment[42, 43].

An important factor which is interconnected with the outcome of macrophage activation is their metabolic state. The interplay between immune cell function and metabolism has taken a prominent place in immunological studies over recent years, leading to the emergence of a new field of research: immunometabolism. Conceptually, functional immune cell activation demands recalibration of cellular metabolism to provide energy in the form of adenosine triphosphate (ATP) and other necessary biosynthetic intermediates. One of the pioneers of cellular physiology was Otto Warburg, who discovered that cancer cell metabolism is skewed towards energy production by glycolysis instead of mitochondrial respiration, even under aerobic conditions[44,45], a process which is since referred to as the 'Warburg effect'. During glycolysis, one molecule of glucose is sequentially converted to two molecules of pyruvate, leading to a net energy production of two ATP (**Figure 2**). Pyruvate is then either used to recycle oxidized nicotinamide adenine dinucleotide (NAD^+) by conversion to lactate, or imported into mitochondria to

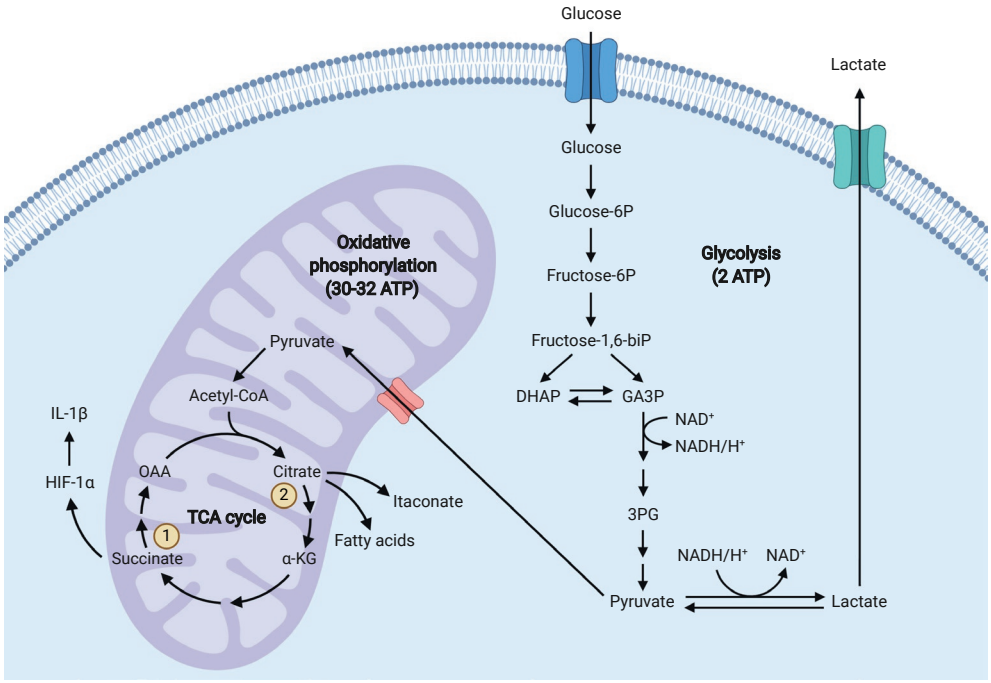

Figure 2: Interference of *Mycobacterium tuberculosis* with phagolysosomal clearance.

Glycolysis yields two molecules of ATP per molecule of glucose. Pyruvate formed after glycolysis is then either converted to lactate (regenerating NAD+) or shuttled to mitochondria to fuel the TCA cycle. Oxidative phosphorylation of pyruvate yields 30-32 ATP per molecule of glucose. Two breaks in the TCA cycle have been reported to occur in pro-inflammatory M1 macrophages: 1) accumulation of succinate was shown to stabilize HIF-1α, leading to IL-1β production; 2) citrate accumulation supports inflammatory action through synthesis of fatty acids and the anti-microbial metabolite itaconate.

form acetyl coenzyme A (acetyl-CoA). Acetyl-CoA subsequently enters the tricarboxylic acid (TCA) cycle, a series of biochemical reactions resulting in the formation of NADH and succinate which are used to produce ~30-32 ATP per glucose molecule during oxidative phosphorylation (OXPHOS). In macrophages and dendritic cells (DCs), a similar metabolic switch towards increased glycolysis was observed after stimulation with TLR ligands[46,47], leading to the hypothesis that induction of the Warburg effect was important for pro-inflammatory activation. Indeed, stimulation with LPS was shown to induce expression of the glycolytic enzyme pyruvate kinase M2 (PKM2)[48], leading to the accumulation of the TCA intermediate succinate, stabilization of hypoxia-inducible factor 1α (HIF-1α) and production of IL-1β, all of which could be abrogated by inhibiting glycolysis using 2-deoxyglucose (2DG)[49]. Due to this redirection of mitochondrial metabolic flux, the TCA cycle has since referred to as being 'broken' in M1 macrophages. In addition to succinate, a second break in the TCA cycle occurs after citrate in these cells[50], which supports

pro-inflammatory M1 macrophage function through synthesis of fatty acids[51] and the immunomodulatory metabolite itaconate [52]. IL-4-induced M2 macrophage polarization, in contrast, is associated with increased OXPHOS and fatty acid oxidation [53] through activation of pathways downstream of signal transducer and activator of transcription 6 (STAT6) and peroxisome proliferator-activated receptor γ (PPAR-γ) coactivator 1β (PGC-1β)[54]. However, metabolic rewiring during myeloid cell activation cannot simply be reduced to glycolysis versus oxidative phosphorylation as it strongly depends on the specific immunogenic stimuli which were used [55,56].

A Warburg-like shift towards glycolysis with associated HIF-1α stabilization and IL-1β production was also observed after *in vitro Mtb* infection in both murine and human macrophages[57,58]. In mice, alveolar macrophages utilizing fatty acid oxidation (FAO) were shown to be more permissive to *Mtb* replication compared to glycolytic interstitial macrophages[59]. Treatment with 2DG, an inhibitor of glycolysis, or etomoxir, an inhibitor of FAO, respectively increased and decreased bacterial growth, functionally linking glycolysis to improved *Mtb* control[57,59]. Interestingly, MDR W-Beijing *Mtb* strains were reported to overexpress phthiocerol dimycocerosate cell wall lipids (PDIMs) which dampen the glycolytic response and IL-1β secretion through induction of IFN-β[60], demonstrating direct mycobacterial modulation of macrophage immunometabolic pathways. In contrast, live *Mtb* infection was reported to result in an overall decreased bioenergetic phenotype in both THP-1 cells and primary human macrophages based on extensive metabolic flux analysis, including a diminished glycolytic rate, reduced lactate production and an increased mitochondrial dependency on fatty acids[61]. It has been proposed that these differences could be the result of a biphasic metabolic response of macrophages to *Mtb* infection, consisting of an early phase characterized by increased glycolysis and production of pro-inflammatory antimicrobial effector molecules, followed by a later phase of adaptation/resolution with intensified oxidative metabolism and decreased antimicrobial responses[62]. However, more definitive evidence for this model will require additional longitudinal studies on the cellular metabolic dynamics during *Mtb* infection.

In addition to glycolysis, *Mtb* infection was found to be associated with other immunometabolic effects, including catabolism of amino acids. Arginine is an important metabolite involved in the antimicrobial response to *Mtb* as it fuels nitric oxide (NO) synthesis through inducible NO synthase (iNOS) in M1 macrophages[63-65], a response which is limited by arginase 1 (Arg1) activity in murine M2 macrophages [66]. In the absence of adequate levels of arginine, citrulline was shown to provide an alternative source of arginine for NO synthesis in response to *Mtb* infection [67-69]. Another key immunomodulatory amino acid is glutamine, the most abundant circulating free amino acid. Glutamine can be directly used as a substrate for the TCA cycle through glutaminolysis, which plays a major role in M2 macrophage polarization[50,70]. Both glutamine depletion and pharmacological inhibition of glutaminolysis decreased *in vitro* cytokine production of PBMCs in response

to *Mtb* lysate, and the transcriptional profile of TB patients and *Mtb*-infected macrophages showed marked changes in glutamine metabolism genes[71], indicating a potential role for glutamine in the response to *Mtb*. Finally, tryptophan conversion to kynurenine by the immunoregulatory enzyme indoleamine 2,3-dioxygenase (IDO1/IDO2) was reported to support *Mtb* infection in a rhesus macaque infection model, as well as in isolated macrophages cocultured with $CD4^+$ T cells [72], possibly by downregulating effector T cell functions [73-75].

In conclusion, it has been unequivocally demonstrated that the functional outcome of macrophage activation is tightly interwoven with cellular metabolic state, and that the latter greatly impacts the outcome of *Mtb* infection. This raises the important question whether dysregulation of these processes as a consequences of metabolic diseases such as type 2 diabetes, a well-known risk factor for TB disease and TB severity, could be related to development TB.

Tuberculosis and diabetes: a reason for concern

The global burden of TB is heavily affected by several serious comorbidities which increase the risk of developing active disease, of which human immunodeficiency virus (HIV) co-infection has been the most prominent during the past decades. HIV infection leads to a 20-fold increased risk of disease reactivation (76). 920,000 people living with HIV developed TB in 2017, and 300,000 people died due to TB-HIV coinfection [2]. Other major TB-associated comorbidities include smoking, alcohol use, exposure to biomass duel and malnourishment [77]. However, an important TB risk factor which has only recently caught the public eye, despite having been previously described centuries ago[78], is diabetes mellitus (DM) [79]. DM has been demonstrated to triple the risk of developing active TB disease (80), and approximately 15% of global TB cases can be attributed to concurrent TB-DM. While the number of TB deaths among HIV-infected patients has declined by 44% since 2000, the number of patients with concurrent TB-DM is predicted to rise dramatically as the number of people living with DM worldwide is estimated to increase by 48% during the coming 25 years, from 425 million people in 2017 to 629 million people in 2045 [81]. The vast majority of this increase is expected to take place in TB-endemic low- and middle-income countries due to changes in lifestyle associated with economic development and urbanization. Furthermore, DM is associated with TB treatment failure and increased drug resistance, while active TB hampers management of glucose control [82]. An international consortium, EC-FP7 supported TANDEM (www.tandem-fp7.eu), was initiated to unravel the relationships and potential mechanisms underlying concurrent TB-DM, and to optimize current treatment regimens and diagnosis [83].

DM is a metabolic disorder which is caused by either a lack of production of the glucose-regulating hormone insulin (type 1/T1DM) or the development of insulin resistance (type 2/T2DM), of which the latter comprises approximately 90% of global cases [81]. Both disease types are characterized by hyperglycemia due to the patient's inability

to control blood glucose levels. Clinical symptoms of DM include recurrent infections, weight gain, polyuria accompanied with excessive thirst, and impaired wound healing. Whereas T1DM is caused by an auto-immune reaction to pancreatic insulin-producing β-cells, the precise mechanisms underlying T2DM are not as clearly defined. Obesity and increasing weight are important risk factors for T2DM development, linking the disease with poor quality nutrition and physical inactivity, as well as family history [84]. This clinical appearance is in stark contrast with the metabolic phenotype of TB patients, which is often accompanied by undernutrition and wasting syndrome [85]. Through its association with obesity, many T2DM patients are also at high risk of cardiovascular complications such as atherosclerosis due to the presence of aberrations in blood lipid levels (dyslipidemia), including hypertriglyceridemia, hypertension and reduced levels high-density lipoprotein (HDL) cholesterol [86].

Insulin resistance does not only occur in the liver and skeletal muscle, the major organs involved in glucose metabolism, but also in the adipose tissue, kidneys, pancreas, gastrointestinal tract, vasculature and brain (87). Generally, the development of insulin resistance precedes T2DM by some time, placing increasing levels of stress on the pancreatic β-cells to produce more insulin which ultimately culminates in β-cell dysfunction[88-90]. Multiple molecular mechanisms of insulin resistance have been described, most of which are related to the phosphatidylinositol 3-kinase (PI3K) pathway [91], a downstream signaling molecule of the insulin receptor. Binding of insulin to its receptor induces phosphorylation of insulin receptor substrates (IRS1 and IRS2), which bind to and activate PI3K [92], subsequently promoting translocation of glucose transporter 4 (GLUT4) protein to the cell surface. It has been demonstrated that increased serine phosphorylation of IRS proteins leads to insulin resistance by inhibiting tyrosine phosphorylation [93] and enhanced IRS protein degradation [94]. Several factors contribute to this increased serine phosphorylation, including activation of protein kinase C (PKC) by accumulating diacylglycerol (DAG) due to ectopic lipid deposition in insulin-sensitive tissues[95], mitochondrial dysfunction[96] and systemic inflammation[97]. Increased infiltration of immune cells and production of pro-inflammatory factors such as TNF-α have been linked to the development of insulin resistance in adipose tissue and in the liver, both in humans and in mice [98]. Specifically, pro-inflammatory M1 macrophages and Th1 cells accumulate in adipose tissue during obesity, while numbers of anti-inflammatory M2 macrophages, Th2 and regulatory T cells (Treg) are reduced [99-101]. Infiltrating macrophages induce adipocyte lipolysis, leading to high local levels of free saturated fatty acids (SFAs) which also contribute to local inflammation and insulin resistance [102], although the causal molecular mechanisms are still a topic of debate [103].

Potential mechanisms responsible for TB-DM disease interactions

While the association between TB and DM has been thoroughly established, the factors underlying this association remain largely unclear. From the above, it is evident that both diseases evoke distinct immunological and metabolic effects. Several studies have examined this interplay by exposing human immune cells to DM-associated conditions such as hyperglycemia, to analyze whether these conditions would disrupt immune responses to *Mtb* infection, while others compared the functional phenotypes of circulating leukocytes in diabetic TB patients or LTBI to normoglycemic controls.

Innate immunity: Monocytes from patients with poorly controlled DM displayed a reduced phagocytic capacity [104, 105]. A similar effect was observed in alveolar macrophages from mice with streptozotocin- or diet-induced diabetes, which showed reduced uptake of mycolic acid-coated beads as well as mycobacteria [106, 107]. This reduced phagocytic potential could negatively impact the capacity of antigen-presenting cells to control bacterial infection and to induce adaptive responses. Furthermore, TB-DM patients had decreased frequencies of circulating intermediate and classical monocytes as well as plasmacytoid and myeloid DCs compared to non-diabetic pulmonary TB patients, which normalized after anti-TB treatment [108, 109]. Other cells of the innate immune system with potentially compromised functions during DM are neutrophils and natural killer (NK) cells. The precise role of neutrophils during TB remains ambivalent, as they potentially contribute to disease protection through mycobacterial killing during early infection, but can also induce tissue damage though release of cytotoxic agents in later stages [110]. Increased neutrophil counts have been reported during TB-DM [111], however isolated neutrophils from DM patients displayed impaired mycobacterial phagocytosis [112]. Moreover, Prada-Medina *et al.* suggested a central role for neutrophilic inflammation in TB-DM based on Bayesian network analysis of cytokine and transcriptomics measurements from Indian patients [113]. NK cells regulate the anti-TB response in several ways, including supporting $CD8^+$ effector T cell responses, enhancing phagocyte bactericidal function through secretion of IFN-γ and IL-22 and releasing antimicrobial effectors such as perforin and granulysin [114]. Importantly, peripheral NK cell numbers negatively correlated with lung inflammation at TB diagnosis, indicative of a relation between circulating NK levels and *Mtb* burden [115]. One study reported expansion of type 1 and type 17 cytokine-producing NK cell populations during TB-DM [116], while others found reduced levels of circulating NK cells in these patients [117]. Interestingly, hyperactive NK cells contributed to inflammation and mortality in a mouse model of TB-DM by inducing IL-6 production in $CD11c^+$ cells [118].

Adaptive immunity: If innate immunity is indeed impaired as a result of TB-DM, this should also profoundly impact the induction of an ensuing adaptive immune response. Indeed, the adaptive response to *Mtb* was described to be further delayed in diabetic mice [119], resulting in increased lung inflammation and bacterial burden [120]. Th1, Th2 and Th17 cytokines were found to be decreased in LTBI with DM compared to normoglycemic controls with LTBI [121]. In contrast, several studies reported hyperinflammatory T cell

response in active TB patients with DM, including elevated TNF-α and IFN-γ production after whole blood stimulation [122], increased circulating levels of pro-inflammatory Th1 and Th17 cytokines [123] and elevated frequencies of central memory $CD4^+$ and $CD8^+$ T cells [124]. While circulating regulatory T cells were reportedly decreased during TB-DM [123], Treg counts were found to be elevated in bronchoalveolar lavage fluid samples of TB-DM patients and associated with increased IL-10 but decreased IFN-γ concentrations [125]. Furthermore, T cells from hyperglycemic mice produced elevated levels of Th1, Th2 and Th17 cytokines due to hyperresponsiveness following T cell receptor ligation [126]. It has been suggested that these elevated inflammatory responses in TB-DM patients could be the result of an increased *Mtb* burden and be responsible for exacerbated lung pathology. Taken together, even though evidence in literature is somewhat conflicting, it can be clearly concluded that concurrent DM is associated with perturbations of both the innate and adaptive response during TB disease.

DM-induced metabolic changes: Mechanistically, several DM-associated factors have been suggested to contribute to dysfunctional immunity. High glucose levels were shown to reduce the phagocytic capacity of but to increase *Mtb* burden in macrophages (127). In addition, hyperglycemia can lead to the formation of advanced glycation end-products (AGEs), which are pathologically glycated proteins or lipids that can modify cellular functions through binding to the AGE receptor (RAGE) [128]. Podell *et al. e.g.* reported that *Mtb* infection increased serum levels of AGEs in guinea pigs [129]. AGEs been shown to induce oxidative stress and ROS production [130, 131], inhibit NO synthesis [132] and increase the expression of scavenger receptors such as CD36 by macrophages. CD36 is involved in the formation of lipid-loaded foam cells through uptake of oxidized low-density lipoprotein (oxLDL) [133, 134]. In resemblance to AGEs, oxLDL is a pathologically modified lipoprotein which is elevated in patients with T2DM as a result of oxidative stress [135, 136]. Interestingly, *Mtb* infection of guinea pigs was associated with oxLDL accumulation in granulomas and increased macrophage scavenger receptor expression, with enhanced mycobacterial replication in macrophages loaded with oxLDL *in vitro* [137]. A major contributor to oxidative stress in DM is reduced synthesis of glutathione (GSH), a tripeptide of glutamate, cysteine and glycine with strong antioxidative properties. GSH levels were found to be reduced in TB patients and infected guinea pigs [138, 139]. As GSH has been demonstrated to have direct antimicrobial effects and to support macrophage control of *Mtb* [140-142], decreased levels of GSH could potentially contribute to TB-DM pathogenesis [143, 144]. Finally, DM and obesity are associated with dysbiosis of the gut microbiome leading to alterations in species which produce short-chain fatty acids (SCFAs) [145, 146], bacterial metabolites with immunomodulatory capacities [147]. TB patients showed increased frequencies of butyrate- and propionate-producing bacteria in the gut [148], and butyrate treatment decreased the production of pro-inflammatory cytokines while increasing IL-10 secretion in PBMCs stimulated with *Mtb* lysate [149], warranting further research into possible dysregulation of gut microbiota during TB-DM.

Specific metabolites: An alternative method which could illuminate potential metabolic components of TB-DM pathophysiology is the use of metabolomics to identify specific alterations in patients associated with disease. Metabolomics is defined as the study of the metabolome, which constitutes the complete collection of small molecule intermediates of metabolism, commonly known as metabolites, within biological samples. The value of this approach is clearly demonstrated by a recent study from Weiner *et al.* that described a prognostic metabolite signature with good predictive power within 5 months of TB diagnosis [150]. Remarkably, specific changes could already be detected at 12 months before onset of disease, which included elevated levels of cortisol. Several studies reported that TB is associated with elevated kynurenine and decreased tryptophan levels [151-153] indicative of increased IDO activity, illustrating how metabolomics measurements can reflect the involvement of relevant immunological mechanisms. However, studies which have analyzed the combined effect of TB and DM on patient metabolic profiles are currently lacking. It would especially be of interest to study measures of lipid metabolism in these patients, as DM and obesity are often associated increased circulating levels of lipids and cholesterol while hypercholesterolemia and dysregulated lipid metabolism are associated with hampered TB immunity and granuloma progression [154-157].

Taken together, concurrent TB-DM and DM-associated metabolic changes are clearly related to alterations in both the innate and the adaptive immune response, although their respective contributions to increased risk of active disease is unclear at present. Extensive analysis of patient materials and *in vitro* infection experiments are required to elucidate the innerworkings of TB-DM comorbidity, which brings us to the outline of this thesis.

Thesis outline

The aim of this thesis was to unravel the pathophysiological mechanisms underlying TB-DM comorbidity, for which we employed several approaches. For the majority of our *in vitro* experiments, we utilized a primary human macrophage model of *Mtb* infection. In **Chapter 2**, we first performed a comparative phenotypic, innate and adaptive functional analysis of various forms/subsets of Mφ2 macrophage polarization. We identify Mφ2b macrophages (polarized in the presence of LPS and immune complexes) as a subset with potent antimycobacterial capabilities. Mφ2b differentiation could be a target for host-directed therapy in intracellular infections.

Secondly, we investigated whether metabolic conditions associated with DM could modulate macrophage activation and function in the context of *Mtb* infection. In **Chapter 3,** we investigated the effect of hyperglycemia on cytokine secretion and *Mtb* infection. We report that PBMCs and macrophages cultured under high glucose conditions showed increased production of several cytokines after stimulation (TNF-α, IL-1β, IL-6 and IL-10). However, *Mtb* survival or outgrowth was not affected by high glucose levels *in vitro*. In **Chapter 4**, we studied the impact of oxLDL on macrophage function and

infection with *Mtb*. We show that oxLDL treatment induced foamy macrophage formation which supported mycobacterial survival through lysosomal cholesterol accumulation and subsequent dysfunction.

Thirdly, we used metabolomics to dissect the relative impact of TB and TB-DM on patients' *ex vivo* metabolic profiles. In **Chapter 5**, we utilized a NMR biomarker profiling platform to analyze circulating levels of amongst others lipids and amino acids in plasma samples of TB, TB-DM and DM patients from South Africa. We find that TB-DM patients possess metabolic characteristics of both diseases and that these are associated with an overall pro-atherogenic plasma lipid profile. We further expand upon the current knowledge of TB metabolomics in **Chapter 6** by measuring plasma concentrations of amines and acylcarnitines in TB and TB-DM patients from Indonesia.

Fourthly, we studied the direct effects of *Mtb* infection on macrophage metabolism to identify new and relevant host metabolic pathways modulated by infection. **Chapter 7** describes the combined results of Seahorse metabolic flux analyses, RNA-seq and (un) targeted cellular metabolomics of *Mtb*-stimulated/-infected macrophages. We find that *Mtb* greatly impacts macrophage metabolism, and highlight specific cellular pathways and metabolic intermediates which are altered as a result of infection.

Finally, the overall results and conclusions of this thesis are summarized and discussed in the concluding **Chapter 8**.

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