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Tuberculosis and diabetes: biomarkers and drug candidates from a host perspective

Doorn, C.L.R. van

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**Tuberculosis and diabetes:
Biomarkers and drug candidates from
a host perspective**

Cassandra Laura Rebecca van Doorn

Tuberculosis and diabetes: Biomarkers and drug candidates from a host perspective

PhD thesis, Leiden University Medical Center, The Netherlands

Author:	Cassandra L.R. van Doorn
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Tuberculosis and diabetes: Biomarkers and drug candidates from a host perspective

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Promotor:

Prof. dr. T.H.M. Ottenhoff

Co-promotor:

Dr. E. Vianello

Leden promotiecommissie:

Prof. dr. A. Geluk

Prof. dr. R.C. Hoeben

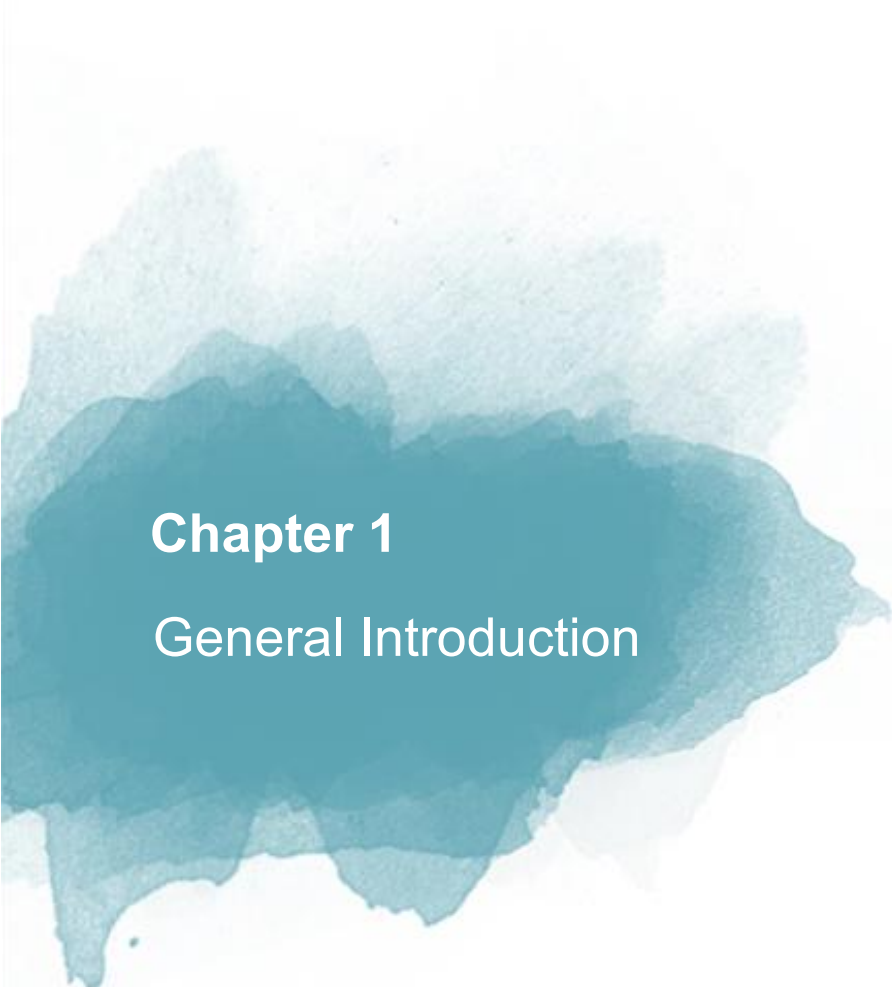
Prof. dr. H.P. Spaink (Department of Animal Sciences and Health, Institute of Biology,
Leiden University)

Dr. E.N.G. Houben (Molecular Microbiology, Amsterdam Institute of Molecular and Life
Sciences, Vrije Universiteit Amsterdam)

Voor papa

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Chapter 1

General Introduction

Cassandra L.R. van Doorn

A history of Tuberculosis

Tuberculosis (TB) is an ancient infectious disease that has long plagued mankind. TB was already described by the ancient Hebrews as 'schachepheth' and by the ancient Greeks as 'phthisis' (1), but the causative agent for TB, *Mycobacterium tuberculosis* (*Mtb*), was not discovered until 1882 by the German Robert Koch (2). *Mtb* is an intracellular bacterial pathogen belonging to the genus of mycobacteria among other pathogenic mycobacteria, including *M. leprae*, *M. ulcerans* and *M. avium*. Using modern DNA techniques, numerous reports have now demonstrated the presence of *Mtb* in ancient skeletons and mummies (3-6), with the oldest sample described until now of estimated 8000-9000 years old (7), indicating the high burden of TB over human history. Death rates by TB were colossal in the 18th and 19th century and began to decline in Western Europe and North America in the late 19th century, and continued to decrease with the discovery of the antibiotic streptomycin and the Bacille Calmette-Guérin (BCG) vaccine (8). Despite its highly variable efficacy, ranging from 0-80%, BCG remains the most widely used vaccine since its discovery in 1921 and the only licensed vaccine against TB (9).

Today, TB remains one of the most deadly infectious diseases, leading to an estimated 10 million TB cases and 1.4 million deaths before the COVID-19 pandemic (*i.e.*, in 2019), especially in low-middle income countries (LMIC) (10). Although the number of TB cases and deaths is declining yearly as a consequence of global progress, the number of TB deaths was increased to 1.5 million in 2020 as a result of the impact of COVID-19, and is expected to have increased even further in 2021 (11).

Threats and challenges for global TB control

Global efforts to eradicate TB target various aspects of the TB epidemic, including increased resources and tools for TB diagnosis, drug-based therapies, novel vaccines, improved health care facilities all accompanied by required funding for research to develop the necessary tools (11). There are several challenges in TB control that need to be tackled. First, the gap between notified TB cases and TB incidence leads to a significant proportion of patients not receiving adequate diagnosis and subsequent treatment. Second, global TB control is threatened by difficult-to-treat infections, due to infections with multidrug-resistant *Mtb* as well as comorbidity with diabetes mellitus (DM). These aspects impact the treatment success rate of TB patients and increase the risk of recurrent TB infections.

Diagnosis

The gap between people who fell ill with TB and the number of people who are diagnosed (and reported) with TB is estimated yearly by the World Health Organization (WHO).

Despite recent advances in TB diagnosis (12), approximately 3 million people with actual TB were estimated not to be diagnosed with active TB or not reported in 2019 (11). Global efforts resulted in an increase of people diagnosed with TB from 5.8 million to 7.1 million between 2012 and 2019, but this trend was reversed to the level of 2012 in 2020 as a consequence of the COVID-19 pandemic, while TB incidence was relatively stable (11). Thus, there is a critical need to increase TB diagnosis.

At present, TB diagnostic tests are based on smear microscopy or molecular diagnostics such as nucleic acid amplification tests (NAATs), along with radiological assessments. However, these traditional diagnostics are critically dependent on sputum samples, which are frequently poorly accessible and of poor quality (13-15). In addition, sputum-based diagnostic tests cannot be used to diagnose extra-pulmonary TB, which requires invasive sampling at site-specific tissues. Alternatively, sputum-independent tuberculin skin tests (TSTs) or Interferon Gamma Release Assays (IGRAs) can be used to detect *Mtb* infection, however, these approaches lack sufficient sensitivity and fail to discriminate active TB from latent *Mtb* infection (LTBI), thereby limiting their diagnostic value in endemic areas (16, 17). Additionally, IGRAs cannot be used to monitor anti-TB therapy response, as no correlation of IGRA results with a clinical response to anti-TB therapy has been demonstrated (18).

Multi-Drug resistant TB

The human immune system evolved defense mechanisms over millions of years in response to invading pathogens, but also pathogens evolved and developed virulence factors to inhibit or even exploit host immunity. The evolutionary arms race between pathogens and their host resulted in sophisticated host immune systems as well as virulence factors, the latter posing increasing challenges to combat pathogens. The discovery of penicillin almost one century ago revolutionized the treatment of infectious diseases caused by bacteria and was rapidly followed by the clinical use of other antibiotics. Since their introduction, antibiotics have protected millions of humans against once-deadly diseases like TB, and without them, *Mtb* would have been estimated to be fatal in 70% of individuals with sputum smear-positive pulmonary TB within 10 years after diagnosis (11). However, over- and mis-usage of antibiotics has provoked microbial resistance against many antibiotics.

Primary resistance is involved following infection with already resistant *Mtb* strains, while secondary or acquired resistance is developed during treatment. Poor treatment compliance of TB patients – fueled by the long duration of standard anti-TB treatment (6 months) and antibiotic-induced side effects – is key to development of acquired resistance. In contrast to the dogma that acquired resistance is predominantly responsible for the occurrence of drug-resistant TB, modelling analysis suggests that primary

resistance is the main driver of the TB resistance epidemic, although there is significant geographical heterogeneity in the contribution of primary versus secondary resistance (19).

Drug-resistant TB has been categorized by the WHO into: isoniazid-resistant TB, rifampicin-resistant (RR)-TB, multidrug-resistant (MDR)-TB, pre-extensively-resistant (pre-XDR)-TB, and extensively-resistant (XDR)-TB (11). MDR-TB is defined as resistance to at least isoniazid and rifampicin. According to current WHO guidelines, MDR-TB patients should be treated with at least four active anti-TB drugs for 18-24 months or longer (11). XDR-TB is MDR-TB that is furthermore resistant to fluoroquinolones and to at least one of the three injectable second-line drugs (amikacin, kanamycin and capreomycin), and totally drug-resistant TB is further resistant to additional drugs.

Drug resistance is often not properly diagnosed, and laboratory techniques for the diagnosis of MDR- and XDR-TB (as reviewed in 20) are severely inaccessible in most high-burden countries. Inappropriate treatment of drug-resistant TB does not only delay treatment responses but may also further amplify resistance to additional drugs. The treatment success rate to first-line regimens is approximately 85%, but only 59% in patients infected with resistant *Mtb* (11). Furthermore, only one in three patients that develop MDR/RR-TB are enrolled to appropriate treatment (11). Mortality rates of XDR-TB are rapidly increasing (Figure 1) (21).

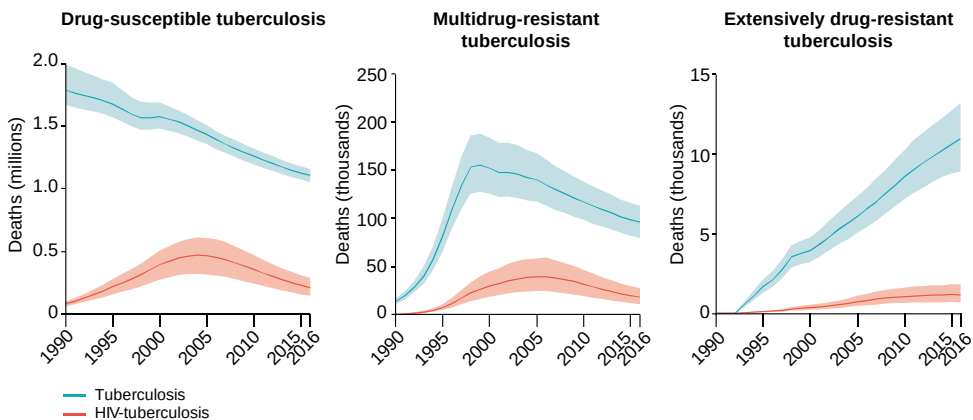


FIGURE 1 | Global tuberculosis mortality by drug-resistance type and HIV status, 1990-2016. Dark lines are estimates and shaded areas are 95% uncertainty intervals. HIV-tuberculosis = tuberculosis in individuals with HIV/AIDS. Multidrug-resistant tuberculosis = multidrug-resistant tuberculosis without extensive drug resistance. Adapted from Kyu *et al.*, 2018 (21).

Despite many efforts in the discovery of novel antibiotics against *Mtb*, only few new drugs are used clinically to treat MDR-TB and XDR-TB, including delamanid, bedaquiline and pretomanid (22, 23). Resistance to delamanid and bedaquiline has already been reported in one patient, and was caused by only few mutations (24), leading to poor perspectives for the future. The discovery of new antimicrobial agents is declining, while the incidence of resistant pathogenic bacteria is on the rise. This prompts for innovative strategies to combat drug-resistant *Mtb* strains.

Tuberculosis and Diabetes

Different risk factors have been established for progression from LTBI to active disease, including malnutrition, smoking, alcohol abuse, coinfection with human immunodeficiency virus (HIV), and DM (11). DM is a chronic disease that is characterized by the inadequate production of or response to insulin, leading to hyperglycaemia. 85-95% of all DM cases is attributed to type-2 diabetes mellitus (T2DM) (25). DM triples the risk for developing active TB and has been associated with poorer clinical outcome following anti-bacterial treatment (26-29). Further, DM increases *Mtb* transmission, as reflected by increased risk of *Mtb* transmission from TB patients with DM or preDM to close contacts (30). This increase in transmission might be caused by delayed mycobacterial clearance in DM patients or by a higher bacillary load. In addition, DM can modify the pharmacokinetics of the anti-TB drugs rifampicin and isoniazid, leading to lower plasma levels of these drugs in TB-DM patients compared to TB patients (31, 32). Furthermore, rifampicin and isoniazid hamper glycaemic control in DM patients by inducing drug-metabolizing enzymes that target most oral anti-diabetic drugs (33). Thus, TB and DM are interconnected at multiple levels.

Approximately 80% of DM patients currently live in low- and middle-income countries, resulting in an increasing geographical overlap with TB prevalence (34). Since global DM prevalence is estimated to increase from 463 million people in 2019 to 700 million in 2045 (35), there is increasing concern about the consequences of the increasing DM prevalence for global TB control. Strikingly, an estimated 3.1% of the TB burden was attributed to DM in 2020 (11). The high incidence of DM among TB patients and vice versa in TB-endemic settings advocates for combined clinical management of TB and DM patients (36, 37).

TB pathogenesis: from infection to disease

Most people that are infected with *Mtb* are able to contain the infection. A quarter of the world's population is estimated to be latently infected with *Mtb*, of which 5-10% will develop active TB during their lifetime (38), that can cause severe lung infection

(pulmonary TB). Inhalation of bacteria-containing aerosols is the main route of infection of *Mtb*, but infection via the digestive tract or damaged skin is also possible. Once an individual is infected via the aerosol route, *Mtb* is ingested by alveolar macrophages (AMs) in the lower respiratory tract, where they are targeted to intracellular compartments called phagosomes (39). During maturation, these phagosomes fuse with early endosomes, late endosomes and lysosomes resulting in an acid and oxidative milieu (40). In the final stage of the maturation process the pathogen is digested efficiently and peptides can be presented to other immune cells such as T cells, via major histocompatibility complex (MHC) molecules. The survival of bacterial pathogens depends on their ability to avoid clearance by the host immune responses. *Mtb* survives within macrophages by interrupting phagosome maturation at an early stage by specialized secretion systems and by blocking acidification of phagolysosomes (41, 42). As a consequence, macrophages can provide a host niche and carbon sources for replication, leading to lung tissue damage. Sites of infection subsequently attract other immune cells, which can ultimately lead to the formation of a primary granuloma which is typical for primary TB (Figure 2). Granulomas are composed of numerous immune cells: macrophages, dendritic cells, neutrophils, natural killer cells, B and T lymphocytes. Granuloma formation is crucial for containment of the *Mtb* infection, but some

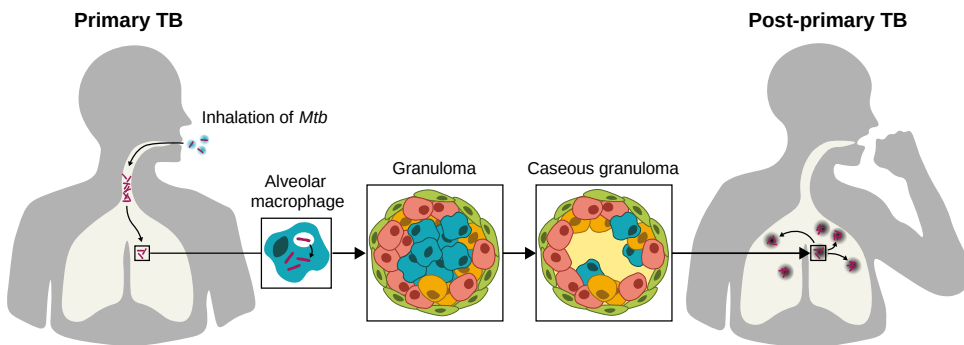


FIGURE 2 | From *Mtb* infection to TB disease. *Mtb* can enter the airways via inhalation and is phagocytosed by AMs. Once engulfed, bacteria can be eradicated by a process called phagosome maturation, or can proliferate inside the macrophage. Infected macrophages attract other immune cells leading to granuloma formation. The granuloma core typically undergoes necrosis leading to a 'caseous' granuloma. Caseous granulomas can form cavities where *Mtb* can proliferate and spread to other parts of the lung, or to other organs, leading to pulmonary TB or extrapulmonary TB, respectively.

granulomas are insufficient in containing the infection, and may even support *Mtb* proliferation and spread (43, 44). The tissue in the center can undergo caseous necrosis and this area is known as a 'Ghon focus'. The encapsulated tissue can undergo fibrosis and calcification to become scar tissue which can be detected on X-rays (called a 'Ranke complex'). *Mtb* can be eradicated, leaving scar tissue, but in immunocompromised individuals, the Ghon focus is reactivated and develops into caseous pneumonia and cavities (post-primary TB) (45). Here, *Mtb* can spread to other parts of the lungs to cause bronchopneumonia or spread to other sites of the body, for example to the skeletal system, kidneys, intestine and brain.

Salmonella infections

Salmonella is a genus of gram-negative bacteria that, like *Mtb*, has evolved mechanisms to combat host intracellular killing mechanisms and replicate within complex phagosomal environments. Salmonella serotypes can be divided into typhoidal and non-typhoidal serotypes, which are both spread via faeco-oral transmission. The severity of the disease caused by Salmonella depends on host factors, which is a shared feature with infections caused by *Mtb*. *Salmonella enterica* serovar Typhi and *S. enterica* serovar Paratyphi represent two typhoidal Salmonella serovars that can cause typhoid fever and paratyphoid fever, respectively. Typhoid and paratyphoid fevers, collectively referred to as enteric fever, are estimated to affect at 14.3 million people annually, and is lethal in approximately 1% of cases (46). Non-typhoidal Salmonella typically causes relatively mild gastroenteritis, but can cause severe disease in immunocompromised patients, children, and the elderly. Multiple highly invasive drug-resistant *S. enterica* serovar Typhimurium (*Stm*) strains have been described (47, 48), demonstrating that in addition to *Mtb*, Salmonella is a major example of antibiotic-resistant pathogens.

Stm can enter macrophages or other (immune) cells via various mechanisms, including the 'trigger' or the 'zipper' mechanism, representing Salmonella-mediated entry (promoted by the Salmonella pathogenicity island-1 type III secretion system) or receptor-mediated entry, respectively (49). Following host cell invasion, *Stm* manifests itself in Salmonella-containing vacuoles (SCVs). *Stm* disrupts the antimicrobial activity of the fused phagolysosome and uses it as a niche for survival (50). Despite differences between *Mtb* and *Stm* in virulence and transmission, both pathogens arrest phagosome maturation, although at different stages of this process (Figure 3).

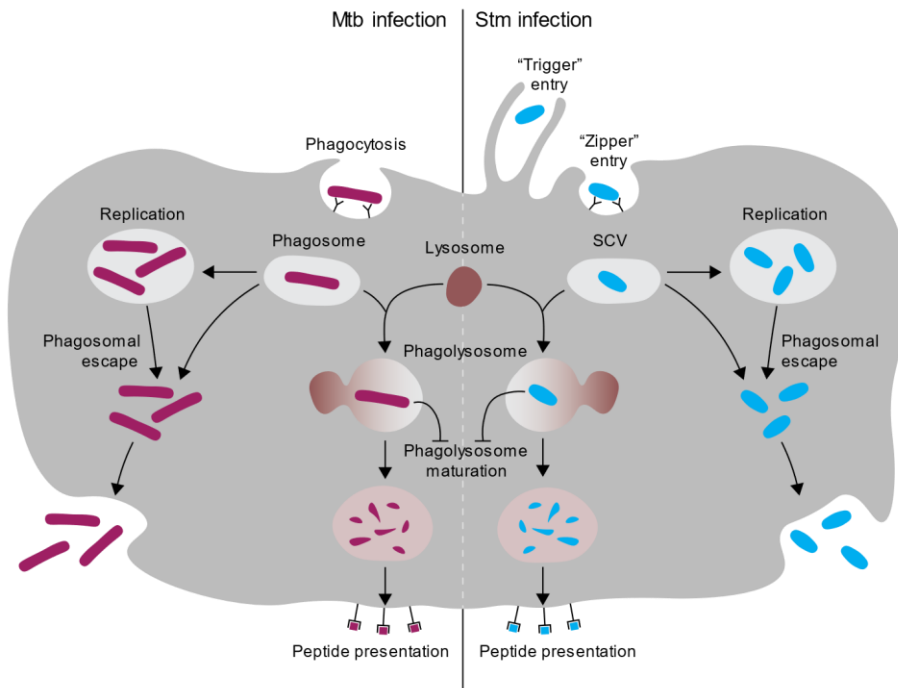


FIGURE 3 | Intracellular bacteria *Mycobacterium tuberculosis* and *Salmonella* adapted to survive and replicate in human macrophages. Bacteria can invade host cells via phagocytosis or bacterium-induced entry and are engulfed into a phagosome or a salmonella-containing vacuole (SCV). Bacteria can be digested via phagolysosome maturation leading to peptide presentation or bacteria can escape the phagosome and replicate intracellularly.

The role of Macrophages during *Mtb* infections

Macrophages are the dominant cell type present in human granulomas, although the majority of macrophages in the TB-granuloma are not infected with *Mtb* (43). Macrophages can elicit different activation states depending on their micro-environment. Classically activated macrophages (M1) induce rapid pro-inflammatory immune responses upon contact with damage- or pathogen-associated molecular patterns, such as lipopolysaccharide (LPS) or bacterial infections. In contrast, alternatively activated macrophages (M2) promote tissue repair and metabolic homeostasis by inducing anti-inflammatory responses.

M1 and M2 phenotypes have been well described in mouse models. While M1s are commonly differentiated by stimulation with interferon-gamma (IFN- γ) and LPS, M2s are differentiated by stimulation with interleukin (IL)-13 and IL-4 (51). Human M1 and M2 macrophages can arise from monocytes stimulated with granulocyte-macrophage colony-

stimulating factor (GM-CSF) or macrophage colony-stimulating factor (M-CSF), respectively (52). Murine and human M1 and M2 produce IL-12 or IL-10 in response to LPS, respectively, whereas surface marker expression is differently characterized between murine and human macrophages (52-58). Martinez and colleagues demonstrated marked differences in the transcriptome and proteome of murine versus human M2, while few conserved macrophage markers were found (59). Further, nitric oxide synthase (iNOS) and arginase-1 (Arg1) are hallmarks for murine M1 and M2 activation, respectively, which was not clearly evident in human M1 and M2 (60). Together, these studies demonstrated fundamental differences in macrophage polarization in murine versus human macrophages in resting cells and upon LPS and IL-4 stimulation.

In addition to their different roles in the innate immune response, murine M1 and M2 macrophages are markedly divergent in their metabolic response to bacterial infections (Figure 4). Murine M1 polarization is characterized by increased glycolysis (61-67), increased flux through the pentose phosphate pathway (PPP) (64-67), suppressed oxidative metabolism (67) and a disrupted tricarboxylic acid (TCA) cycle after citrate and after succinate (66). Increased glycolysis is characterized by increased glucose consumption and lactate production *in the presence of oxygen*, demonstrating a metabolic parallel between macrophage activation and tumor immunology (*i.e.*, the Warburg effect).

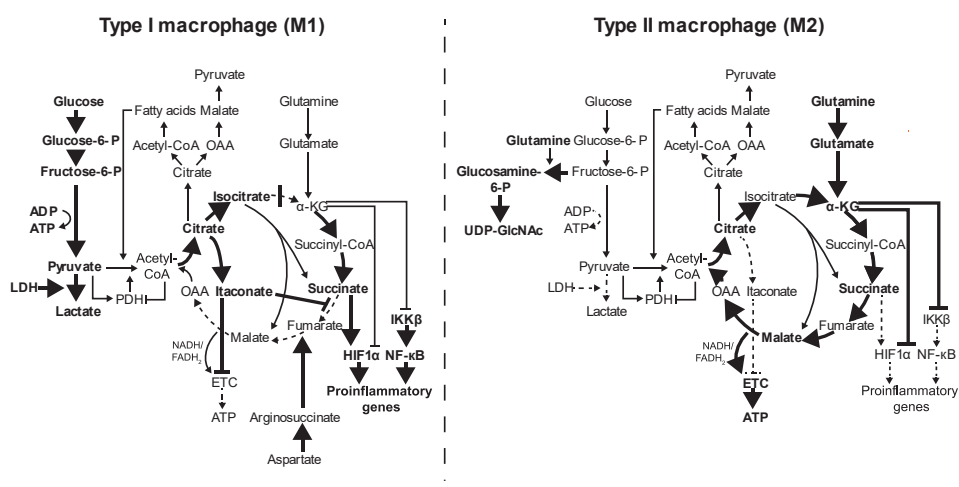


FIGURE 4 | Metabolic divergence in murine macrophages that are polarized towards M1 or M2. Bold arrows indicate pathway upregulation and dashed arrows indicate pathway downregulation. Protein associations are adapted from following publications: (61-69).

The metabolic shift is promoted by the transcription factor hypoxia-inducible factor 1 alpha (HIF-1 α), which increases the expression of glycolytic genes (68). Murine M2 polarization is characterized by oxidative phosphorylation (OxPhos) (61-63), a complete TCA cycle, fatty acid oxidation (FAO) (62), glutamine metabolism (66, 69) and the hexosamine biosynthesis pathway (HSP) (66). Upregulation of the glutamine metabolic pathway results in supply of the TCA cycle via α -ketoglutarate (α KG), a key metabolite in the induction of M2 activation and suppression of M1 activation in mouse BMDMs (69).

Although macrophages are often described as M1 versus M2 polar extremes, many studies have shown that external stimuli can induce a wider range of macrophage differentiation-states and subsets (70, 71). In TB patients, macrophages with M2 characteristics predominated in granulomas, whereas both macrophages with a M1 or M2 phenotype were found in non-granulomatous lung tissue (72). In steady state, human AMs were found to express M1/M2 characteristics simultaneously, suggesting that they can rapidly polarize upon exposure to external stimuli (73).

TB-DM interactions in macrophages

TB patients with DM comorbidity display a qualitative and quantitative altered immune response of immune cell subsets (*e.g.*, T helper type 1, T helper type 17 and natural killer cells) compared to patients with uncomplicated TB, which could contribute to increased TB susceptibility and disease (74-76). The mechanisms underlying TB-DM comorbidity at the cellular level remain largely unknown, and studies on how the macrophage response might be perturbed by DM are scarce. Given the prominent role of macrophages in *Mtb* control, it is likely that DM-induced functional modifications could negatively impact host immunity against *Mtb*. To better control the TB-DM co-epidemic, fundamental research that focuses on the bidirectional interactivity between DM-associated molecular processes and immune responses to *Mtb* is urgently needed.

Energy metabolism plays a role in the development and progression of DM, indicated by a decreased OxPhos rate and increased lipoprotein levels (77-80). Such metabolic effects could modulate immune cell function, and could affect the availability of host nutrients. For instance, *in vitro* accumulation of oxidized low-density lipoprotein (oxLDL), which has been associated with DM, impairs *Mtb* control by human macrophages and was associated with decreased *Mtb* localization to functional lysosomes (81). Further, it has been demonstrated that monocytes and monocyte-derived macrophages (MDMs) from T2DM patients displayed a perturbed inflammatory immune response to *Mtb* and a decreased *Mtb* killing ability compared to MDMs from healthy donors, indicating a direct link between TB and DM at the cellular level (34, 82-85). Specifically, MDMs obtained from T2DM patients showed a diminished cytokine response (IL-12, IL-8, IL-1 β) and lower

expression of macrophage activation markers (HLA-DR, CD86, CD163, CD14 and CD80) upon *in vitro* *Mtb* infection, potentially leading to a deficient T cell response (34, 84, 85). Furthermore, *Mtb*-induced nitric oxide production was impaired in AMs from (type 1 and type 2) diabetic rats compared to AMs from non-diabetic rats, suggesting reduced immune activation in these AMs (86, 87). In addition, other studies showed decreased *Mtb* phagocytosis and control by AMs of mice with streptozotocin-induced or diet-induced diabetes when compared to control mice (88-91).

Reduced phagocytosis and macrophage activation could delay adaptive immune responses, possibly contributing to enhanced TB susceptibility in patients with concomitant DM. In agreement with this hypothesis, recent data suggest that DM decreases *in vitro* antigen presentation by M1s obtained from TB patients (92). However, *in vitro* stimulation of PBMCs with increasing concentrations of glucose, mimicking hyperglycaemia, was not associated with differences in *Mtb* killing or outgrowth, suggesting that hyperglycaemia alone cannot explain the reduced killing capacity of macrophages obtained from TB-DM patients versus patients with uncomplicated TB (93).

Improving TB diagnostics: transcriptomic-based biomarkers

The transcriptome represents the genetic code transcribed into RNA transcripts in a cell, a population of cells or in an individual. Any physiological condition, for example a disease, can affect the relative abundance of transcripts, and analysis of the transcriptome is therefore often used to study the immune response to a disease. RNA expression analysis is a powerful tool to reveal fundamental host responses that are associated with TB, but may also represent an appealing non-sputum-based approach to diagnose active TB and to monitor the response to TB treatment (94).

The TB transcriptome has been well described, and numerous gene signatures that distinguish TB from LTBI and healthy controls (HCs) have been reported (Table 1). T2DM patients display persistent low grade inflammation, and the blood transcriptome of T2DM patients is characterized by upregulation of genes involved in inflammation, fatty acid metabolism and T-cell signaling (95, 96). T2DM patients have been excluded from most TB transcriptomic studies, although DM can interfere with the blood transcriptome of TB patients, which can lead to reduced performance of TB biosignatures (97). For TB diagnostics, it is crucial to identify gene signatures that are applicable in patients with TB irrespective of their glycaemic or DM status. Transcriptomic-based biomarkers are likely to improve TB control by enhancing clinical surveillance and by accelerating decision making to embark alternative or additional treatment strategies. Furthermore, TB-related gene signatures could facilitate the evaluation of novel vaccine and drug trials.

TABLE 1 | Published TB transcriptomes.

SIGNATURE	YEAR	POPULATION	STUDY DESIGN	SOURCE	PLATFORM	REF
JACOBSEN	2007	Germany	TB vs LTBI, HC	PBMC	Microarray, RT-qPCR	(98)
BERRY	2010	UK, South Africa	TB vs LTBI, HC, OD	WB	Microarray	(99)
MAERTZDORF	2011	South Africa	TB vs LTBI, HC	WB	Microarray, RT-qPCR	(100)
BLOOM	2012	South Africa	TB (LONG)	WB	Microarray	(101)
OTTENHOFF	2012	Indonesia	TB vs HC, TB (LONG)	PBMC	Microarray	(102)
JOOSTEN	2012	The Gambia, Paraguay	TB vs LTBI, HC, TB (LONG)	WB	dcRT-MLPA	(103)
KAFOROU	2013	South Africa, Malawi	TB vs LTBI, OD (HIV)	WB	Microarray	(104)
BLOOM	2013	UK	TB vs OD	WB	Microarray	(105)
VERHAGEN	2013	Warao Amerindian children	TB vs LTBI, HC	WB	Microarray, RT-qPCR	(106)
ANDERSON	2014	African children (South Africa, Malawi, Kenya)	TB vs LTBI, OD	WB	Microarray	(107)
SWEENEY	2016	Multicohort study	TB vs LTBI, OD	WB	Microarray	(108)
ZAK	2016	South Africa	LTBI to TB (LONG)	WB	RNA-Seq, RT-qPCR	(109)
ROE	2016	UK	TB vs HC, TB (LONG)	WB	Microarray	(110)
THOMPSON	2017	South Africa	TB (LONG)	WB	RNA-Seq, RT-qPCR	(111)
GJØEN	2017	Indian children	TB vs HHC, symptomatic non-TB	WB	dcRT-MLPA	(112)
GEBREMICAEL	2018	Ethiopia (HIV+)	TB vs LTBI, HC	WB	dcRT-MLPA	(113)
WARSINKE	2018	Brazil, South Africa	TB vs LTBI	WB	RNA-Seq	(114)
SULIMAN	2018	South Africa, the Gambia, Ethiopia, Uganda	HHC to TB (LONG)	WB	RNA-Seq, RT-qPCR	(115)
SIVAKUMARAN	2020	India	TB (LONG)	WB	dcRT-MLPA	(116)
BUREL	2021	UK	TB (LONG)	WB	Microarray	(117)
GLIDDON	2021	South Africa, Malawi	TB vs LTBI, OD	WB	RT-dPCR	(118)

Abbreviations: HC, healthy control; HHC, household contact; LONG, longitudinal; LTBI, latent tuberculosis infection; OD, other diseases; PBMC, peripheral blood mononuclear cell; RT-dPCR, reverse transcription digital polymerase chain reaction; RT-qPCR, reverse transcription quantitative real-time polymerase chain reaction; TB, tuberculosis; WB, whole blood.

Novel treatment strategies against an ancient microbe: host-directed therapy

Innate and adaptive immune systems play a crucial role during the course of *Mtb* infection, as reflected by the majority of hosts developing LTBI following *Mtb* infection. For this reason, targeting host immunity poses an alternative and innovative opportunity for the treatment of active TB. Progress in understanding pathogen-host interactions during TB pathogenesis permitted the development of host-directed therapy (HDT) as an approach to target host factors instead of pathogenic factors directly. HDTs can act against *Mtb* by activating host pathways that induce bacterial clearance or by inhibiting bacterial replication, and can be effective against LTBI and MDR/XDR-TB, and potentially also against difficult-to-target dormant, non-replicating bacteria (119, 120). HDT is less prone to de-novo resistance compared to antibiotics-based therapy, since it requires the pathogen to acquire considerable numbers of mutations to evade complex host defense mechanisms (120, 121).

Although HDT offers the potential to treat viral and bacterial infections, the clinical use of HDTs is yet very limited. An example of HDT used in the clinic is Maraviroc (Selzentry/Celsentri; Pfizer), a CCR5 inhibitor used as HIV-1 therapy (122). For the treatment of *Mtb* infections, several HDTs are being tested in clinical trials, including statins, vitamin D3, and metformin (121). The potential of HDTs to reduce intracellular *Mtb* has been demonstrated *in vitro* in murine and human macrophages, and HDTs are therefore proposed as adjunctive therapy to antibiotics, to shorten treatment regimens and to improve treatment success of difficult-to-treat *Mtb* infections (*i.e.*, co-morbidity with DM or MDR-TB and XDR-TB).

Scope and outline of this thesis

In this thesis we describe our work regarding the identification of novel biomarkers, host targets and candidate pharmacological compounds for the development of therapies against various intracellular bacterial infections, focusing primarily on the interplay between TB and DM.

First, we conducted two longitudinal cohort studies in South Africa and Indonesia, and we applied unbiased and selective transcriptomic approaches to identify novel biomarker profiles in TB patients with concomitant DM or hyperglycaemia. We described functional differences in the immune response to anti-TB treatment in patients with or without DM and TB-related hyperglycaemia and we showed that despite these differences, gene signatures can be obtained to predict the anti-TB response irrespective of DM/glycaemia severity (**Chapter 2**). Further, we showed that the clinical outcome of anti-TB treatment was associated with immunological pathways that could be detected in

whole blood using transcriptomics-based approaches, and we were able to identify a transcriptomic biomarker signature to predict anti-TB treatment outcome in our cohorts (South Africa and Indonesia) as well as in an external validation cohort (India) (**Chapter 3**).

Second, we performed experiments with standardized *in vitro* cellular infection models for drug discovery, focusing primarily on infections with *Mtb*, but also on infections with the human pathogen *Stm*. Based on published studies that showed a perturbed response to *Mtb* in MDMs from T2DM patients compared to MDMs from healthy controls (34), we hypothesized that small molecule inhibitors that interfere with DM-pathways at the cellular level may be able to redirect macrophage function in response to *Mtb* infection. Such chemical compounds could be valuable tools as part of immunomodulatory HDT regimens. In **Chapter 4**, we identified poly (ADP) ribose polymerases (PARPs) as potential host targets during *Mtb* infections and we describe the clinical potential of PARP inhibitors for the treatment of *Mtb* infections. In **Chapter 5**, we describe the role of central metabolic pathways and, in more detail, the role of metabolic key regulator pyruvate dehydrogenase kinases during *Mtb* and *Stm* infections in human macrophages.

The major findings of the above studies are summarized and discussed in **Chapter 6**.

References

1. Barberis I, Bragazzi NL, Galluzzo L, Martini M. The history of tuberculosis: from the first historical records to the isolation of Koch's bacillus. *Journal of preventive medicine and hygiene*. 2017;58(1):E9.
2. Koch R. The etiology of tuberculosis. *Mittheilungen aus dem Kaiserlichen Gesundheitsamte*. 1884;2:1-88.
3. Bos KI, Harkins KM, Herbig A, Coscolla M, Weber N, Comas I, et al. Pre-Columbian mycobacterial genomes reveal seals as a source of New World human tuberculosis. *Nature*. 2014;514(7523):494-7.
4. Nicklisch N, Maixner F, Ganslmeier R, Friederich S, Dresely V, Meller H, et al. Rib lesions in skeletons from early neolithic sites in Central Germany: on the trail of tuberculosis at the onset of agriculture. *American journal of physical anthropology*. 2012;149(3):391-404.
5. Zink AR, Sola C, Reischl U, Grabner W, Rastogi N, Wolf H, et al. Characterization of *Mycobacterium tuberculosis* complex DNAs from Egyptian mummies by spoligotyping. *Journal of clinical microbiology*. 2003;41(1):359-67.
6. Zink A, Haas CJ, Reischl UDO, Szeimies U, Nerlich AG. Molecular analysis of skeletal tuberculosis in an ancient Egyptian population. *Journal of medical microbiology*. 2001;50(4):355-66.
7. Hershkovitz I, Donoghue HD, Minnikin DE, Besra GS, Lee OYC, Gernaey AM, et al. Detection and molecular characterization of 9000-year-old *Mycobacterium tuberculosis* from a Neolithic settlement in the Eastern Mediterranean. *PLoS one*. 2008;3(10):e3426.
8. Daniel TM. The impact of tuberculosis on civilization. *Infectious Disease Clinics*. 2004;18(1):157-65.
9. Brewer TF. Preventing tuberculosis with bacillus Calmette-Guerin vaccine: a meta-analysis of the literature. *Clinical Infectious Diseases*. 2000;31(Supplement_3):S64-S7.
10. World Health Organization. Global tuberculosis report 2020. Geneva: World Health Organization; 2020. Licence: CC BY-NC-SA 3.0 IGO. 2020.

11. World Health Organization. Global tuberculosis report 2021. Geneva: World Health Organization. Licence: CC BY-NC-SA 3.0 IGO. 2021.
12. Acharya B, Acharya A, Gautam S, Ghimire SP, Mishra G, Parajuli N, et al. Advances in diagnosis of Tuberculosis: an update into molecular diagnosis of *Mycobacterium tuberculosis*. *Molecular biology reports*. 2020;47(5):4065-75.
13. Hernández P, Punchak M, Camacho M, Hepple P, McNerney R. Investigating the quality of expectorated sputum for tuberculosis diagnosis in Bolivia. *The International Journal of Tuberculosis and Lung Disease*. 2015;19(9):1065-7.
14. Bhat J, Rao V, Muniyandi M, Yadav R, Karforma C, Luke C. Impact of sputum quality and quantity on smear and culture positivity: findings from a tuberculosis prevalence study in central India. *Transactions of the Royal Society of Tropical Medicine and Hygiene*. 2014;108(1):55-6.
15. Meyer AJ, Atuheire C, Worodria W, Kizito S, Katamba A, Sanyu I, et al. Sputum quality and diagnostic performance of GeneXpert MTB/RIF among smear-negative adults with presumed tuberculosis in Uganda. *PloS one*. 2017;12(7):e0180572.
16. Sester M, Sotgiu G, Lange C, Giehl C, Girardi E, Migliori GB, et al. Interferon- γ release assays for the diagnosis of active tuberculosis: a systematic review and meta-analysis. *European Respiratory Journal*. 2011;37(1):100-11.
17. Syed Ahamed Kabeer B, Raman B, Thomas A, Perumal V, Raja A. Role of QuantiFERON-TB gold, interferon gamma inducible protein-10 and tuberculin skin test in active tuberculosis diagnosis. *PLoS One*. 2010;5(2):e9051.
18. Clifford V, He Y, Zufferey C, Connell T, Curtis N. Interferon gamma release assays for monitoring the response to treatment for tuberculosis: a systematic review. *Tuberculosis*. 2015;95(6):639-50.
19. Kendall EA, Fofana MO, Dowdy DW. Burden of transmitted multidrug resistance in epidemics of tuberculosis: a transmission modelling analysis. *The Lancet Respiratory Medicine*. 2015;3(12):963-72.
20. Dheda K, Gumbo T, Maartens G, Dooley KE, McNerney R, Murray M, et al. The epidemiology, pathogenesis, transmission, diagnosis, and management of multidrug-resistant, extensively drug-resistant, and incurable tuberculosis. *The lancet Respiratory medicine*. 2017;5(4):291-360.
21. Kyu HH, Maddison ER, Henry NJ, Ledesma JR, Wiens KE, Reiner Jr R, et al. Global, regional, and national burden of tuberculosis, 1990–2016: results from the Global Burden of Diseases, Injuries, and Risk Factors 2016 Study. *The Lancet Infectious Diseases*. 2018;18(12):1329-49.
22. Zumla A, Memish ZA, Maeurer M, Bates M, Mwaba P, Al-Tawfiq JA, et al. Emerging novel and antimicrobial-resistant respiratory tract infections: new drug development and therapeutic options. *The Lancet Infectious Diseases*. 2014;14(11):1136-49.
23. Keam SJ. Pretomanid: first approval. *Drugs*. 2019;79(16):1797-803.
24. Bloemberg GV, Gagneux S, Böttger EC. Acquired resistance to bedaquiline and delamanid in therapy for tuberculosis. *The New England journal of medicine*. 2015;373(20):1986.
25. Tuomilehto J. The emerging global epidemic of type 1 diabetes. *Current diabetes reports*. 2013;13(6):795-804.
26. Baker MA, Harries AD, Jeon CY, Hart JE, Kapur A, Lönnroth K, et al. The impact of diabetes on tuberculosis treatment outcomes: a systematic review. *BMC medicine*. 2011;9(1):1-15.
27. Jeon CY, Murray MB. Diabetes mellitus increases the risk of active tuberculosis: a systematic review of 13 observational studies. *PLoS medicine*. 2008;5(7):e152.
28. Lin C-H, Kuo S-C, Hsieh M-C, Ho S-Y, Su I-J, Lin S-H, et al. Effect of diabetes mellitus on risk of latent TB infection in a high TB incidence area: a community-based study in Taiwan. *BMJ open*. 2019;9(10):e029948.

29. Arriaga MB, Araújo-Pereira M, Barreto-Duarte B, Nogueira B, Freire MVC, Queiroz AT, et al. The Effect of Diabetes and Prediabetes on Antituberculosis Treatment Outcomes: A Multicenter Prospective Cohort Study. *The Journal of Infectious Diseases*. 2022;225(4):617-26.
30. Arriaga MB, Rocha MS, Nogueira BM, Nascimento V, Araújo-Pereira M, Souza AB, et al. The Effect of Diabetes and Prediabetes on *Mycobacterium tuberculosis* Transmission to Close Contacts. *The Journal of Infectious Diseases*. 2021;224(12):2064-72.
31. Mtabho CM, Semvua HH, van den Boogaard J, Irongo CF, Boeree MJ, Colbers A, et al. Effect of diabetes mellitus on TB drug concentrations in Tanzanian patients. *Journal of Antimicrobial Chemotherapy*. 2019;74(12):3537-45.
32. Nijland HMJ, Ruslami R, Stalenhoef JE, Nelwan EJ, Alisjahbana B, Nelwan RHH, et al. Exposure to rifampicin is strongly reduced in patients with tuberculosis and type 2 diabetes. *Clinical Infectious Diseases*. 2006;43(7):848-54.
33. Ruslami R, Aarnoutse RE, Alisjahbana B, Van Der Ven AJAM, Van Crevel R. Implications of the global increase of diabetes for tuberculosis control and patient care. *Tropical Medicine & International Health*. 2010;15(11):1289-99.
34. Lopez-Lopez N, Martinez AGR, Garcia-Hernandez MH, Hernandez-Pando R, Castañeda-Delgado JE, Lugo-Villarino G, et al. Type-2 diabetes alters the basal phenotype of human macrophages and diminishes their capacity to respond, internalise, and control *Mycobacterium tuberculosis*. *Memórias do Instituto Oswaldo Cruz*. 2018;113(4).
35. Saeedi P, Petersohn I, Salpea P, Malanda B, Karuranga S, Unwin N, et al. Global and regional diabetes prevalence estimates for 2019 and projections for 2030 and 2045: Results from the International Diabetes Federation Diabetes Atlas. *Diabetes research and clinical practice*. 2019;157:107843.
36. McAllister SM, Koesoemadinata RC, Santoso P, Soetedjo NNM, Kamil A, Permana H, et al. High tuberculosis incidence among people living with diabetes in Indonesia. *Transactions of The Royal Society of Tropical Medicine and Hygiene*. 2020;114(2):79-85.
37. Ugarte-Gil C, Alisjahbana B, Ronacher K, Riza AL, Koesoemadinata RC, Malherbe ST, et al. Diabetes mellitus among pulmonary tuberculosis patients from 4 tuberculosis-endemic countries: the TANDEM study. *Clinical Infectious Diseases*. 2020;70(5):780-8.
38. Cohen A, Mathiasen VD, Schön T, Wejse C. The global prevalence of latent tuberculosis: a systematic review and meta-analysis. *European Respiratory Journal*. 2019;54(3).
39. Philips JA, Ernst JD. Tuberculosis pathogenesis and immunity. *Annual Review of Pathology: Mechanisms of Disease*. 2012;7:353-84.
40. Flannagan RS, Cosío G, Grinstein S. Antimicrobial mechanisms of phagocytes and bacterial evasion strategies. *Nature Reviews Microbiology*. 2009;7(5):355-66.
41. Zhai W, Wu F, Zhang Y, Fu Y, Liu Z. The immune escape mechanisms of *Mycobacterium tuberculosis*. *International journal of molecular sciences*. 2019;20(2):340.
42. Pethe K, Swenson DL, Alonso S, Anderson J, Wang C, Russell DG. Isolation of *Mycobacterium tuberculosis* mutants defective in the arrest of phagosome maturation. *Proceedings of the National Academy of Sciences*. 2004;101(37):13642-7.
43. Flynn JL, Chan J, Lin PL. Macrophages and control of granulomatous inflammation in tuberculosis. *Mucosal immunology*. 2011;4(3):271-8.
44. Ramakrishnan L. Revisiting the role of the granuloma in tuberculosis. *Nature Reviews Immunology*. 2012;12(5):352-66.
45. Hunter RL. The pathogenesis of tuberculosis: the early infiltrate of post-primary (adult pulmonary) tuberculosis: a distinct disease entity. *Frontiers in immunology*. 2018;9:2108.
46. Stanaway JD, Reiner RC, Blacker BF, Goldberg EM, Khalil IA, Troeger CE, et al. The global burden of typhoid and paratyphoid fevers: a systematic analysis for the Global Burden of Disease Study 2017. *The Lancet Infectious Diseases*. 2019;19(4):369-81.

47. Kingsley RA, Msefula CL, Thomson NR, Kariuki S, Holt KE, Gordon MA, et al. Epidemic multiple drug resistant *Salmonella* Typhimurium causing invasive disease in sub-Saharan Africa have a distinct genotype. *Genome research*. 2009;19(12):2279-87.
48. Mather AE, Phuong TLT, Gao Y, Clare S, Mukhopadhyay S, Goulding DA, et al. New variant of multidrug-resistant *Salmonella enterica* serovar Typhimurium associated with invasive disease in immunocompromised patients in Vietnam. *MBio*. 2018;9(5):e01056-18.
49. Velge P, Wiedemann A, Rosselin M, Abed N, Boumart Z, Chaussé A-M, et al. Multiplicity of *Salmonella* entry mechanisms, a new paradigm for *Salmonella* pathogenesis. *Microbiologyopen*. 2012;1(3):243-58.
50. Amer AO, Swanson MS. A phagosome of one's own: a microbial guide to life in the macrophage. *Current opinion in microbiology*. 2002;5(1):56-61.
51. Martinez FO, Sica A, Mantovani A, Locati M. Macrophage activation and polarization. *Front Biosci*. 2008;13(1):453-61.
52. Verreck FAW, de Boer T, Langenberg DML, Hoeve MA, Kramer M, Vaisberg E, et al. Human IL-23-producing type 1 macrophages promote but IL-10-producing type 2 macrophages subvert immunity to (myco) bacteria. *Proceedings of the National Academy of Sciences*. 2004;101(13):4560-5.
53. Jablonski KA, Amici SA, Webb LM, Ruiz-Rosado JdD, Popovich PG, Partida-Sanchez S, et al. Novel markers to delineate murine M1 and M2 macrophages. *PLoS one*. 2015;10(12):e0145342.
54. Orecchioni M, Ghosheh Y, Pramod AB, Ley K. Macrophage polarization: different gene signatures in M1 (LPS+) vs. classically and M2 (LPS-) vs. alternatively activated macrophages. *Frontiers in immunology*. 2019;10:1084.
55. Fernando MR, Reyes JL, Iannuzzi J, Leung G, McKay DM. The pro-inflammatory cytokine, interleukin-6, enhances the polarization of alternatively activated macrophages. *PLoS one*. 2014;9(4):e94188.
56. Heemskerk MT, Korbé CJ, Esselink JJ, dos Santos CC, van Veen S, Gordijn IF, et al. Repurposing diphenylbutylpiperidine-class antipsychotic drugs for host-directed therapy of *Mycobacterium tuberculosis* and *Salmonella enterica* infections. *Scientific Reports*. 2021;11(1):19634.
57. Reales-Calderón JA, Aguilera-Montilla N, Corbí ÁL, Molero G, Gil C. Proteomic characterization of human proinflammatory M1 and anti-inflammatory M2 macrophages and their response to *Candida albicans*. *Proteomics*. 2014;14(12):1503-18.
58. Vijayan V, Pradhan P, Braud L, Fuchs HR, Gueler F, Motterlini R, et al. Human and murine macrophages exhibit differential metabolic responses to lipopolysaccharide-A divergent role for glycolysis. *Redox biology*. 2019;22:101147.
59. Martinez FO, Helming L, Milde R, Varin A, Melgert BN, Draijer C, et al. Genetic programs expressed in resting and IL-4 alternatively activated mouse and human macrophages: similarities and differences. *Blood, The Journal of the American Society of Hematology*. 2013;121(9):e57-e69.
60. Galván-Peña S, O'Neill LAJ. Metabolic reprogramming in macrophage polarization. *Frontiers in immunology*. 2014;5:420.
61. Rodríguez-Prados J-C, Través PG, Cuenca J, Rico D, Aragonés J, Martín-Sanz P, et al. Substrate fate in activated macrophages: a comparison between innate, classic, and alternative activation. *The Journal of Immunology*. 2010;185(1):605-14.
62. Vats D, Mukundan L, Odegaard JI, Zhang L, Smith KL, Morel CR, et al. Oxidative metabolism and PGC-1 β attenuate macrophage-mediated inflammation. *Cell metabolism*. 2006;4(1):13-24.
63. Huang SC-C, Everts B, Ivanova Y, O'Sullivan D, Nascimento M, Smith AM, et al. Cell-intrinsic lysosomal lipolysis is essential for alternative activation of macrophages. *Nature immunology*. 2014;15(9):846-55.

64. Freemerman AJ, Johnson AR, Sacks GN, Milner JJ, Kirk EL, Troester MA, et al. Metabolic reprogramming of macrophages: glucose transporter 1 (GLUT1)-mediated glucose metabolism drives a proinflammatory phenotype. *Journal of Biological Chemistry*. 2014;289(11):7884-96.
65. Haschemi A, Kosma P, Gille L, Evans CR, Burant CF, Starkl P, et al. The sedoheptulose kinase CARKL directs macrophage polarization through control of glucose metabolism. *Cell metabolism*. 2012;15(6):813-26.
66. Jha AK, Huang SC-C, Sergushichev A, Lampropoulou V, Ivanova Y, Loginicheva E, et al. Network integration of parallel metabolic and transcriptional data reveals metabolic modules that regulate macrophage polarization. *Immunity*. 2015;42(3):419-30.
67. Tannahill GM, Curtis AM, Adamik J, Palsson-McDermott EM, McGettrick AF, Goel G, et al. Succinate is an inflammatory signal that induces IL-1 β through HIF-1 α . *Nature*. 2013;496(7444):238-42.
68. Kelly B, O'Neill LA. Metabolic reprogramming in macrophages and dendritic cells in innate immunity. *Cell research*. 2015;25(7):771-84.
69. Liu P-S, Wang H, Li X, Chao T, Teav T, Christen S, et al. α -ketoglutarate orchestrates macrophage activation through metabolic and epigenetic reprogramming. *Nature immunology*. 2017;18(9):985-94.
70. Mantovani A, Sica A, Sozzani S, Allavena P, Vecchi A, Locati M. The chemokine system in diverse forms of macrophage activation and polarization. *Trends in immunology*. 2004;25(12):677-86.
71. Xue J, Schmidt SV, Sander J, Draffehn A, Krebs W, Quester I, et al. Transcriptome-based network analysis reveals a spectrum model of human macrophage activation. *Immunity*. 2014;40(2):274-88.
72. Huang Z, Luo Q, Guo Y, Chen J, Xiong G, Peng Y, et al. *Mycobacterium tuberculosis*-induced polarization of human macrophage orchestrates the formation and development of tuberculous granulomas in vitro. *PloS one*. 2015;10(6):e0129744.
73. Mitsi E, Kamng'ona R, Rylance J, Solórzano C, Jesus Reiné J, Mwandumba HC, et al. Human alveolar macrophages predominately express combined classical M1 and M2 surface markers in steady state. *Respiratory research*. 2018;19(1):1-4.
74. Kumar NP, Sridhar R, Banurekha VV, Jawahar MS, Nutman TB, Babu S. Expansion of pathogen-specific T-helper 1 and T-helper 17 cells in pulmonary tuberculosis with coincident type 2 diabetes mellitus. *The Journal of infectious diseases*. 2013;208(5):739-48.
75. Kumar NP, Sridhar R, Nair D, Banurekha VV, Nutman TB, Babu S. Type 2 diabetes mellitus is associated with altered CD 8+ T and natural killer cell function in pulmonary tuberculosis. *Immunology*. 2015;144(4):677-86.
76. Kumar NP, Sridhar R, Banurekha VV, Jawahar MS, Fay MP, Nutman TB, et al. Type 2 diabetes mellitus coincident with pulmonary tuberculosis is associated with heightened systemic type 1, type 17, and other proinflammatory cytokines. *Annals of the American Thoracic Society*. 2013;10(5):441-9.
77. Patti ME, Butte AJ, Crunkhorn S, Cusi K, Berria R, Kashyap S, et al. Coordinated reduction of genes of oxidative metabolism in humans with insulin resistance and diabetes: Potential role of PGC1 and NRF1. *Proceedings of the National Academy of Sciences*. 2003;100(14):8466-71.
78. Olsson AH, Yang BT, Hall E, Taneera J, Salehi A, Nitert MD, et al. Decreased expression of genes involved in oxidative phosphorylation in human pancreatic islets from patients with type 2 diabetes. *European journal of endocrinology*. 2011;165(4):589.
79. Marin MT, Dasari PS, Tryggestad JB, Aston CE, Teague AM, Short KR. Oxidized HDL and LDL in adolescents with type 2 diabetes compared to normal weight and obese peers. *Journal of Diabetes and its Complications*. 2015;29(5):679-85.
80. Vrieling F, Ronacher K, Kleynhans L, Van Den Akker E, Walzl G, Ottenhoff THM, et al. Patients with concurrent tuberculosis and diabetes have a pro-atherogenic plasma lipid profile. *EBioMedicine*. 2018;32:192-200.

81. Vrieling F, Wilson L, Rensen PCN, Walzl G, Ottenhoff THM, Joosten SA. Oxidized low-density lipoprotein (oxLDL) supports *Mycobacterium tuberculosis* survival in macrophages by inducing lysosomal dysfunction. *PLoS pathogens*. 2019;15(4):e1007724.
82. Restrepo BI, Twahirwa M, Rahbar MH, Schlesinger LS. Phagocytosis via complement or Fc-gamma receptors is compromised in monocytes from type 2 diabetes patients with chronic hyperglycemia. *PloS one*. 2014;9(3):e92977.
83. Gomez DI, Twahirwa M, Schlesinger LS, Restrepo BI. Reduced *Mycobacterium tuberculosis* association with monocytes from diabetes patients that have poor glucose control. *Tuberculosis*. 2013;93(2):192-7.
84. Valtierra-Alvarado MA, Delgado JEC, Ramírez-Talavera SI, Lugo-Villarino G, Dueñas-Arteaga F, Lugo-Sánchez A, et al. Type 2 diabetes mellitus metabolic control correlates with the phenotype of human monocytes and monocyte-derived macrophages. *Journal of Diabetes and its Complications*. 2020;34(11):107708.
85. Valtierra-Alvarado MA, Lugo-Villarino G, Dueñas-Arteaga F, González-Contreras BE, Lugo-Sánchez A, Castañeda-Delgado JE, et al. Impact of type 2 diabetes on the capacity of human macrophages infected with *Mycobacterium tuberculosis* to modulate monocyte differentiation through a bystander effect. *Immunology and Cell Biology*. 2021;99(10):1026-39.
86. Sugawara I, Mizuno S. Higher susceptibility of type 1 diabetic rats to *Mycobacterium tuberculosis* infection. *The Tohoku journal of experimental medicine*. 2008;216(4):363-70.
87. Sugawara I, Yamada H, Mizuno S. Pulmonary tuberculosis in spontaneously diabetic goto kakizaki rats. *The Tohoku journal of experimental medicine*. 2004;204(2):135-45.
88. Alim MA, Kupz A, Sikder S, Rush C, Govan B, Ketheesan N. Increased susceptibility to *Mycobacterium tuberculosis* infection in a diet-induced murine model of type 2 diabetes. *Microbes and infection*. 2020;22(8):303-11.
89. Martens GW, Arian MC, Lee J, Ren F, Greiner D, Kornfeld H. Tuberculosis susceptibility of diabetic mice. *American journal of respiratory cell and molecular biology*. 2007;37(5):518-24.
90. Martinez N, Ketheesan N, West K, Vallerskog T, Kornfeld H. Impaired recognition of *Mycobacterium tuberculosis* by alveolar macrophages from diabetic mice. *The Journal of infectious diseases*. 2016;214(11):1629-37.
91. Vallerskog T, Martens GW, Kornfeld H. Diabetic mice display a delayed adaptive immune response to *Mycobacterium tuberculosis*. *The Journal of Immunology*. 2010;184(11):6275-82.
92. Restrepo BI, Khan A, Singh VK, de-Leon E, Aguillón-Durán GP, Ledezma-Campos E, et al. Human monocyte-derived macrophage responses to *M. tuberculosis* differ by the host's tuberculosis, diabetes or obesity status, and are enhanced by rapamycin. *Tuberculosis*. 2021;126:102047.
93. Lachmandas E, Vrieling F, Wilson LG, Joosten SA, Netea MG, Ottenhoff TH, et al. The effect of hyperglycaemia on in vitro cytokine production and macrophage infection with *Mycobacterium tuberculosis*. *PloS one*. 2015;10(2):e0117941.
94. Warsinske H, Vashisht R, Khatri P. Host-response-based gene signatures for tuberculosis diagnosis: A systematic comparison of 16 signatures. *PLoS medicine*. 2019;16(4):e1002786.
95. Manoel-Caetano FS, Xavier DJ, Evangelista AF, Takahashi P, Collares CV, Puthier D, et al. Gene expression profiles displayed by peripheral blood mononuclear cells from patients with type 2 diabetes mellitus focusing on biological processes implicated on the pathogenesis of the disease. *Gene*. 2012;511(2):151-60.
96. Grayson BL, Wang L, Aune TM. Peripheral blood gene expression profiles in metabolic syndrome, coronary artery disease and type 2 diabetes. *Genes & Immunity*. 2011;12(5):341-51.
97. Eckold C, Kumar V, Weiner J, Alisjahbana B, Riza A-L, Ronacher K, et al. Impact of Intermediate Hyperglycemia and Diabetes on Immune Dysfunction in Tuberculosis. *Clinical Infectious Diseases*. 2021;72(1):69-78.

98. Jacobsen M, Repsilber D, Gutschmidt A, Neher A, Feldmann K, Mollenkopf HJ, et al. Candidate biomarkers for discrimination between infection and disease caused by *Mycobacterium tuberculosis*. *Journal of molecular medicine*. 2007;85(6):613-21.
99. Berry MPR, Graham CM, McNab FW, Xu Z, Bloch SAA, Oni T, et al. An interferon-inducible neutrophil-driven blood transcriptional signature in human tuberculosis. *Nature*. 2010;466(7309):973-7.
100. Maertzdorf J, Repsilber D, Parida SK, Stanley K, Roberts T, Black G, et al. Human gene expression profiles of susceptibility and resistance in tuberculosis. *Genes & Immunity*. 2011;12(1):15-22.
101. Bloom CI, Graham CM, Berry MPR, Wilkinson KA, Oni T, Rozakeas F, et al. Detectable changes in the blood transcriptome are present after two weeks of antituberculosis therapy. *PLoS one* 2012;7(10): e46191.
102. Ottenhoff THM, Dass RH, Yang N, Zhang MM, Wong HEE, Sahiratmadja E, et al. Genome-wide expression profiling identifies type 1 interferon response pathways in active tuberculosis. *PLoS one*. 2012;7(9): e45839.
103. Joosten SA, Goeman JJ, Sutherland JS, Opmeer L, De Boer KG, Jacobsen M, et al. Identification of biomarkers for tuberculosis disease using a novel dual-color RT–MLPA assay. *Genes & Immunity*. 2012;13(1):71-82.
104. Kaforou M, Wright VJ, Oni T, French N, Anderson ST, Bangani N, et al. Detection of tuberculosis in HIV-infected and-uninfected African adults using whole blood RNA expression signatures: a case-control study. *PLoS medicine*. 2013;10(10):e1001538.
105. Bloom CI, Graham CM, Berry MPR, Rozakeas F, Redford PS, Wang Y, et al. Transcriptional blood signatures distinguish pulmonary tuberculosis, pulmonary sarcoidosis, pneumonias and lung cancers. *PLoS one*. 2013;8(8):e70630.
106. Verhagen LM, Zomer A, Maes M, Villalba JA, Del Nogal B, Eleveld M, et al. A predictive signature gene set for discriminating active from latent tuberculosis in Warao Amerindian children. *BMC genomics*. 2013;14(1):1-11.
107. Anderson ST, Kaforou M, Brent AJ, Wright VJ, Banwell CM, Chagaluka G, et al. Diagnosis of childhood tuberculosis and host RNA expression in Africa. *New England Journal of Medicine*. 2014;370(18):1712-23.
108. Sweeney TE, Braviak L, Tato CM, Khatri P. Genome-wide expression for diagnosis of pulmonary tuberculosis: a multicohort analysis. *The Lancet Respiratory Medicine*. 2016;4(3):213-24.
109. Zak DE, Penn-Nicholson A, Scriba TJ, Thompson E, Suliman S, Amon LM, et al. A prospective blood RNA signature for tuberculosis disease risk. *J Lancet*. 2016;387(10035):2312.
110. Roe JK, Thomas N, Gil E, Best K, Tsaliki E, Morris-Jones S, et al. Blood transcriptomic diagnosis of pulmonary and extrapulmonary tuberculosis. *JCI insight*. 2016;1(16).
111. Thompson EG, Du Y, Malherbe ST, Shankar S, Braun J, Valvo J, et al. Host blood RNA signatures predict the outcome of tuberculosis treatment. *Tuberculosis*. 2017;107:48-58.
112. Gjøen JE, Jenum S, Sivakumaran D, Mukherjee A, Macaden R, Kabra SK, et al. Novel transcriptional signatures for sputum-independent diagnostics of tuberculosis in children. *Scientific reports*. 2017;7(1):1-9.
113. Gebremicael G, Kassa D, Quinten E, Alemayehu Y, Gebreegziavier A, Belay Y, et al. Host gene expression kinetics during treatment of tuberculosis in HIV-coinfected individuals is independent of highly active antiretroviral therapy. *The Journal of infectious diseases*. 2018;218(11):1833-46.
114. Warsinske HC, Rao AM, Moreira FMF, Santos PCP, Liu AB, Scott M, et al. Assessment of validity of a blood-based 3-gene signature score for progression and diagnosis of tuberculosis, disease severity, and treatment response. *JAMA network open*. 2018;1(6):e183779-e.

115. Suliman S, Thompson EG, Sutherland J, Weiner 3rd J, Ota MOC, Shankar S, et al. Four-gene pan-African blood signature predicts progression to tuberculosis. *American journal of respiratory and critical care medicine*. 2018;197(9):1198-208.
116. Sivakumaran D, Jenum S, Vaz M, Selvam S, Ottenhoff THM, Haks MC, et al. Combining host-derived biomarkers with patient characteristics improves signature performance in predicting tuberculosis treatment outcomes. *Communications biology*. 2020;3(1):1-10.
117. Burel JG, Singhanian A, Dubelko P, Muller J, Tanner R, Parizotto E, et al. Distinct blood transcriptomic signature of treatment in latent tuberculosis infected individuals at risk of developing active disease. *Tuberculosis*. 2021;131:102127.
118. Gliddon HD, Kaforou M, Alikian M, Habgood-Coote D, Zhou C, Oni T, et al. Identification of Reduced Host Transcriptomic Signatures for Tuberculosis Disease and Digital PCR-Based Validation and Quantification. *Frontiers in immunology*. 2021;12:433.
119. Mandal S, Njikan S, Kumar A, Early JV, Parish T. The relevance of persisters in tuberculosis drug discovery. *Microbiology*. 2019;165(5):492-9.
120. Kiliñ G, Saris A, Ottenhoff THM, Haks MC. Host-directed therapy to combat mycobacterial infections. *Immunological reviews*. 2021;301(1):62-83.
121. Kaufmann SHE, Dorhoi A, Hotchkiss RS, Bartenschlager R. Host-directed therapies for bacterial and viral infections. *Nature reviews Drug discovery*. 2018;17(1):35-56.
122. Woollard SM, Kanmogne GD. Maraviroc: a review of its use in HIV infection and beyond. *Drug design, development and therapy*. 2015;9:5447.

Chapter 2

Impaired resolution of blood transcriptomes through tuberculosis treatment with diabetes comorbidity

Clare Eckold*, Cassandra L.R. van Doorn*, Rovina Ruslami, Katharina Ronacher, Anca-Lelia Costache, Suzanne van Veen, Ji-Sook Lee, Vinod Kumar, Sarah Kerry-Barnard, Stephanus T. Malherbe, Léanie Kleynhans, Kim Stanley, Simone A. Joosten, Julia A. Critchley, Philip C. Hill, Reinout van Crevel, Cisca Wijmenga, Mariëlle C. Haks, Mihai Loana, Bakti Alisjahbana, Gerhard Walzl, Tom H.M. Ottenhoff, Hazel M. Dockrell, Eleonora Vianello[#] and Jacqueline M. Cliff[#], on behalf of the TANDEM Consortium

**These authors contributed equally*

[#]These authors contributed equally

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Abstract

Background

People with diabetes are more likely to develop tuberculosis (TB) and to have poor TB treatment outcomes than those without. We previously showed that blood transcriptomes in people with TB-diabetes (TB-DM) co-morbidity have excessive inflammatory and reduced interferon responses at TB diagnosis. It is unknown whether this persists through treatment, potentially underlying adverse outcomes.

Methods

Pulmonary TB patients were recruited in South Africa, Indonesia and Romania, and classified as having TB-DM, TB with prediabetes, TB-related hyperglycaemia or uncomplicated TB, based on glycated haemoglobin (HbA1c) concentration at TB diagnosis and after 6 months of TB treatment. Gene expression in blood samples collected at diagnosis and at regular intervals throughout treatment was measured by unbiased RNASeq and targeted Multiplex Ligation-dependent Probe Amplification.

Results

Gene expression was modulated by TB treatment in all groups but to different extents, such that differences remained in people with TB-DM relative to TB-only throughout, including genes involved in innate responses, anti-microbial immunity and the inflammasome. People with prediabetes or with TB-related hyperglycaemia had gene expression more similar to people with TB-DM than TB-only throughout treatment. The overall pattern of change was similar across clinical groups irrespective of glycaemic index, permitting models predictive of TB treatment to be developed.

Conclusions

The exacerbated transcriptome changes seen in TB-DM take longer to resolve during TB treatment, indicating that prolonged treatment or host-directed therapy may be needed to improve TB treatment outcomes. Development of transcriptome-based biomarker signatures of TB-treatment response should include people with diabetes to be useful across populations.

Introduction

Diabetes mellitus (DM) negatively impacts TB control by increasing the risk of *Mycobacterium tuberculosis* infection [1] and of progression to active TB disease three-fold [2, 3]. The growing prevalence of DM, particularly in countries with high TB burden, means DM now underlies around 15% of TB cases globally [4], accounting for 10% of TB deaths in HIV-negative people. Concomitant DM negatively impacts TB-treatment outcomes, with increased risk of delayed sputum conversion, relapse, treatment failure and death: relative risk for each poor outcome is ~2 to ~5 in meta-analyses [5, 6]. It is unknown whether extending standard TB treatment would improve outcome for TB-DM comorbid patients, or whether alternative treatment is required, such as host-directed therapy.

Worldwide DM prevalence is ~463 million people, estimated to rise to 700 million by 2045 [7]. The majority of people have type-2 DM, caused by a reduction in insulin's ability to control target cell metabolism triggering an increase in insulin production, pancreatic damage through exhaustion, and impaired glucose tolerance. There is a spectrum from normal through to full DM via intermediate hyperglycaemia (IH). People with IH are more likely to develop DM in the future [8]. HbA1c concentration can indicate an individual's position on this spectrum [8]. Infectious diseases, including TB, can cause temporary stress hyperglycaemia, which carries a higher risk of adverse events than longer-term pre-diabetes [9]. TB-induced stress hyperglycaemia also makes DM diagnosis difficult: some people with apparent newly-diagnosed DM at TB diagnosis no longer reach DM diagnostic criteria after TB treatment [10]. TB incidence and TB-DM treatment outcomes are worse in people with poorly controlled DM with higher HbA1c concentrations [11].

People with TB-DM comorbidity have altered immunity compared to uncomplicated TB, with both innate and adaptive immune responses affected [12]. In plasma, various inflammatory cytokines such as IL-1 β , IL-17A, IFN γ and TNF α are more elevated in TB-DM [13, 14] and TB-pre-diabetes [15] than in uncomplicated TB. People with TB-DM have more circulating Th1 and Th17 cells and fewer Tregs. In uncomplicated TB, peripheral immune responses typically resolve to normal levels during successful TB treatment [14]. In contrast, the excessive inflammatory plasma cytokine responses in TB-DM are still evident after treatment completion [16], and dendritic cell, monocyte [17] and T cell differentiation [18] aberrations are still present at 2 months, although resolved by 6 months, indicating a delayed response to TB treatment in TB-DM patients.

Transcriptomic technologies have delineated altered peripheral immunity in TB in multiple studies, revealing an enhanced circulating inflammatory and type 1 interferon response [19-22]. With successful TB treatment, the transcriptomic signature is rapidly downregulated, has largely diminished after two months of treatment and mostly

disappears by 12 months [20, 22, 23], mirroring clinical resolution and chest X-ray improvement; however transcriptomes do not fully resolve with poor TB treatment outcome [24]. We recently showed [25] that DM comorbidity, as well as IH, significantly affects the TB diagnosis biosignature, causing an enhanced inflammatory but reduced interferon response. The impact of DM on transcriptomes through TB treatment has not been described. The aim of this study was to determine whether transcriptomic biosignatures resolve normally in TBDM, or whether changes during TB treatment are kinetically or qualitatively different to those in uncomplicated TB.

Methods

Patient recruitment and classification.

Newly diagnosed patients with bacteriologically confirmed pulmonary TB, with or without concomitant DM, were recruited in three locations: Bandung, Indonesia (UNPAD), Cape Town, South Africa (SUN) and Craiova, Romania (UMFCV), as part of the TANDEM project [26]. Exclusion criteria were multi-drug-resistant TB, HIV positivity, pregnancy, other serious co-morbidity or corticosteroid use. All participants gave written informed consent. The study was approved by LSHTM Observational Research Ethics Committee (6449/July2013), SUN Health Research Ethics Committee (N13/05/064/July2013), UNPAD Health Research Ethics Committee (377/UN6.C2.1.2/ KEPK/PN/2012), and UMFCV Committee of Ethics and Academic and Scientific Deontology (94/September2013).

All TB patients underwent first line TB treatment according to WHO guidelines. Most patients diagnosed with DM received local standard of care treatment, and medication taken was noted. A TB-DM subgroup within the Indonesian cohort had intensive HbA1c monitoring as part of a pragmatic randomised control trial, with DM medication changed accordingly [27]. Participants were classified by DM/glycaemia status at TB diagnosis and after 6 months TB treatment (Supplementary Table S1). The “TB-DM” group included patients with both pre-existing and newly diagnosed DM (Supplementary Table S2) Newly diagnosed TB-DM had laboratory HbA1c test $\geq 6.5\%$ with confirmatory HbA1c test $\geq 6.5\%$ or fasting blood glucose ≥ 7 mmol/L at TB diagnosis [26, 28], followed by a further HbA1c test $\geq 6.5\%$ after 6 months of TB treatment. Patients whose HbA1c test results were $\geq 5.7\%$ and $< 6.5\%$ at both TB diagnosis and at 6 months were deemed to have pre-diabetes (“TB-preDM”). Patients whose HbA1c result was $\geq 5.7\%$ at TB diagnosis but below $< 5.7\%$ at 6 months were deemed to have TB-related intermediate hyperglycaemia at TB diagnosis (“TBrel-IH”). All groups were evenly sex balanced, except for male predominance in TB-PreDM. Age ranges were similar across groups. In the Indonesian TB-DM group, there was a highly significant decrease in HbA1c through TB treatment, likely due to intensive DM follow-up (Supplementary Figure S1 & Supplementary Table S1); this was not evident in

South Africa or Romania.

Sample collection and RNA extraction

Venous blood samples (2.5ml) were collected into PAXgene Blood RNA Tubes (PreAnalytiX) from TB patients prior to TB treatment initiation (W0) and at intervals through treatment (W2,4,8,16,26) up to 12 months post diagnosis (W52), and stored at -80oC prior to analysis. Total RNA was extracted using RNeasy spin columns (Qiagen) and quantified by Nanodrop (Agilent).

Unbiased whole genome RNA-Seq

A detailed description of RNA-Seq analysis is given in Supplementary Methods. Samples were processed using the poly-A tail Bioscientific NEXTflex-Rapid-Directional mRNA-seq method and single-end sequenced. Longitudinal differential gene expression analyses were performed on normalised data using the MaSigPro (v1.62.0) [29] package in R. MaSigPro follows a two-step regression method to find genes with significant temporal expression changes and significant differences between groups. Modular analysis was performed on genes that were differentially expressed between clinical groups.

Targeted gene expression profiling

Targeted gene expression profiling was performed using dual-color Reverse-Transcriptase Multiplex Ligation-dependent Probe Amplification (dcRT-MLPA) [30]: a detailed description is given in Supplementary Methods. Primers and half-probes were designed for 4 housekeeping genes and 144 selected key immune TB-related genes (Supplementary Table S3). Longitudinal differentially expressed genes (DEGs) within groups were identified using linear mixed models. Signatures with the best discriminatory capability were identified using logistic regression with lasso regularization (glmnet R package).

Results

Global longitudinal transcriptomes in TB-DM

Gene expression was determined in venous blood by RNA-Seq in a subgroup of study participants from the four TB patient clinical groups (Supplementary Table S1; Supplementary Figure S1). The molecular degree of perturbation (MDP) of gene expression in individual samples from patients with TB-only or TB-DM over time was calculated relative to mean gene expression at diagnosis in TB-only (Figure 1). As expected, there were gene expression changes during TB treatment in the TB-only group, evident by week 2 and continuing throughout treatment. Global gene expression was perturbed in patients with TB-DM relative to TB-only at TB diagnosis, and while there was some resolution through time, the changes were reduced such that global gene expression in the TB-DM group remained different to the TB-only group at all time points (Figure 1).

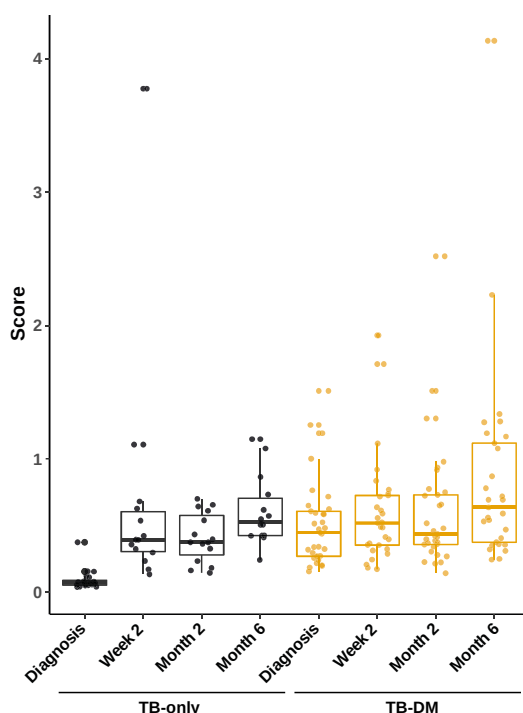


FIGURE 1 | Molecular Degree of Perturbation Plots representing change in global gene expression in blood relative to patients with TB-only at TB diagnosis. Gene expression was determined by RNA-Seq of whole venous blood from pulmonary TB patients from all three clinical locations with (TB-DM: n=34) or without (TB-only: n= 18) concomitant diabetes, at TB diagnosis and during TB treatment. The bars show the median and 1.5*inter-quartile range.

The MaSigPro analysis package identified 167 genes with significantly different changed expression between TB-DM and TB-only groups through TB treatment, in the combined dataset from South Africa, Indonesia and Romania. Hierarchical clustering of these genes based on similar expression patterns yielded 9 clusters (Figure 2; Supplementary Table S4). Clusters which were more highly expressed in TB-DM patients throughout treatment (clusters 1,2,4&8) were enriched for genes involved in the innate immune response, IL-4 signalling, protein dimerisation and neutrophil chemotaxis, determined using the DAVID Functional Annotation Tool [31] (Table 1). Cluster 6 exhibited divergence between TB and TB-DM patients only at week 8 of treatment: this cluster was enriched for genes involved in anti-viral and IFN signalling responses. Clusters more highly expressed in TB-only patients (clusters 5,7,9) were smaller and enriched for alternative splice variants and immunoglobulins.

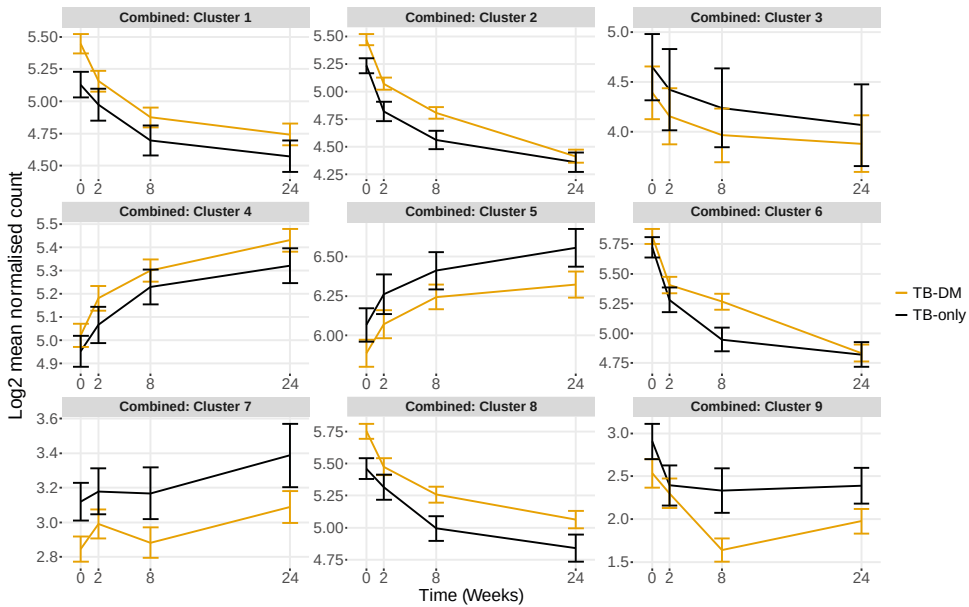


FIGURE 2 | MaSigPro analysis of change in gene expression through TB treatment in blood samples from patients in all 3 populations combined (South Africa, Indonesia and Romania). MaSigPro identified genes that behave similarly between patient groups using hierarchical clustering. Results are shown for log-transformed normalised count for the TB-only group or TB-DM. Bars show mean $\pm$ 1 SEM. Data were filtered to remove lowly abundant transcripts prior to analysis.

Aberrant longitudinal transcriptomes in TB patients with intermediate hyperglycaemia

Previously [25] we showed gene expression in TBrel-IH is more similar to people with diagnosed DM than with TB-only at TB diagnosis. We repeated the MaSigPro analyses separately for South Africa and Indonesia, combining all those patients with raised glycaemic indices but not DM at TB diagnosis, to determine how transcriptomes changed through TB treatment in intermediate groups (Supplementary Figure S2). In South Africa, the analysis resulted in 1,179 transcripts separated into three hierarchical clusters, which changed through treatment differently across clinical groups (Supplementary Figure S2A; Supplementary Table S5), with the combined intermediate group behaving more similarly to TB-DM. Similar results were obtained with the Indonesian cohort, with 2,354 transcripts across 4 hierarchical clusters behaving differently between clinical groups (Supplementary Figure S2B; Supplementary Table S6).

A core list of 102 genes overlapped between MaSigPro analyses for the combined cohort from Romania, South Africa and Indonesia, and from the latter two populations separately (Supplementary Figure S3; Supplementary Table S7). Gene ontology and pathway analyses of this core list using the g:profiler webtool revealed functional enrichment of genes involved in the immune response, response to biotic

TABLE 1 | Clusters of genes differentially expressed between TB-DM and TB-only patients in MaSigPro analysis of the combined RNA-Seq dataset from South Africa, Indonesia and Romania.

Cluster	Overall pattern	Number of transcripts	Gene Function				Top Non-redundant Functions from DAVID [#]
			Protein Coding	Processed Transcript	Pseudo-gene	Regulatory RNAs [*]	
1	Higher in TB-DM throughout; decreasing with time	27	19	0	2	6	Innate Immunity Antimicrobial RAGE receptor binding
2	Higher in TB-DM throughout; decreasing with time	50	28	5	4	13	IPAF inflammasome IL-4 signalling Transmembrane helices
3	Lower in TB-DM throughout; decreasing with time	6	6	0	0	0	Disulphide bond Inflammation/fibrosis
4	Higher in TB-DM throughout; increasing with time	17	11	3	0	3	Coiled coil Protein homo-dimerisation
5	Lower in TB-DM throughout; increasing with time	9	8	1	0	0	Collagen-binding Alternative splicing phosphoprotein
6	Higher in TB-DM at week 8, otherwise similar	28	22	1	0	5	GTPase activity Anti-viral defence IFN γ signalling
7	Lower in TB-DM throughout; rising end treatment	10	4	1	0	5	Splice variant
8	Higher in TB-DM throughout; decreasing with time	16	13	2	0	1	Secreted Neutrophil chemotaxis Transmembrane helix
9	Lower in TB-DM at week 8 and 24, otherwise similar	4	3	0	0	1	Immunoglobulins

[#]DAVID [31] analysis of GO terms BP, MF, CC direct UP-Keywords^{*}Retained introns, Antisense, LncRNA, miRNA, nonsense-mediated decay, sense overlapping, sense intronic, snoRNA,

stimuli, and gene products localising to intracellular vesicles (Supplementary Figure S4). We hypothesised there would be differences between the TB-preDM and TBrel-IH groups, due to persistence or resolution of hyperglycaemia through TB treatment; however, both TB-preDM and TBrel-IH patients responded more similarly to TB-DM than to TB-only patients (Figure 3). Longitudinal mixed effects model analysis of mean expression within core gene list clusters showed highly significant changes across all four clinical groups throughout treatment, with differences between the groups in larger gene clusters (Supplementary Table S8). Importantly, there was no interaction between clinical group and time, showing there was resolution of expression in all groups through treatment, albeit from different starting points and at different rates.

Modular analysis of differentially expressed genes (DEGs)

DEGs identified in MaSigPro analyses were used in modular analyses to understand biological differences between clinical groups in South Africa and Indonesia (Supplementary Table 9 and 10 respectively). The most statistically significant modules were investigated further by calculating their modular activity in TB-DM relative to TB-only through time. The top module in both populations was immune activation, which was upregulated in TB-DM compared to TB-only throughout treatment. In both populations, different modules fluctuated between TB-DM and TB, and behaved inversely to one another through treatment (Figure 4).

Impact of DM on TB treatment response using targeted gene expression profiling

We performed targeted profiling of TB-relevant immune gene expression in an expanded cohort from South Africa with more intensive sampling, using dcRT-MLPA (Supplementary Table S11A). At baseline, the overall MDP was similar in all study groups compared to healthy controls (Supplementary Figure S5A); Partial Least Squares – Discriminant Analysis separated DM-only patients from other groups including healthy controls, suggesting distinct genes are perturbed in DM-only (Supplementary Figure S5B; [25]). Gene expression was strongly correlated between TB-only and TB-DM, TB-preDM or TBrel-IH, but with some outlier genes which were affected by glycaemic status (Figure 5A). There were more DEGs relative to healthy controls in the TB-DM, TB-preDM and TBrel-IH groups than TB-only, at TB diagnosis and throughout treatment (Figure 5B). Normalisation of expression of genes such as *GNLY* and *GBP1* occurred by 2 weeks in the TB-only group but was delayed in TBDM, TB-preDM and TBrel-IH. Results from targeted dcRT-MLPA analysis were thus in accordance with the global RNA-Seq analysis.

Longitudinal MDP analysis in the South African and Indonesian cohorts indicated the magnitude of transcriptomic response to TB treatment was dependent on diabetes/glycaemia with TB-DM patients displaying the largest gene expression perturbation over time (Figure 6A, Supplementary Figure S6A). Gene expression changes

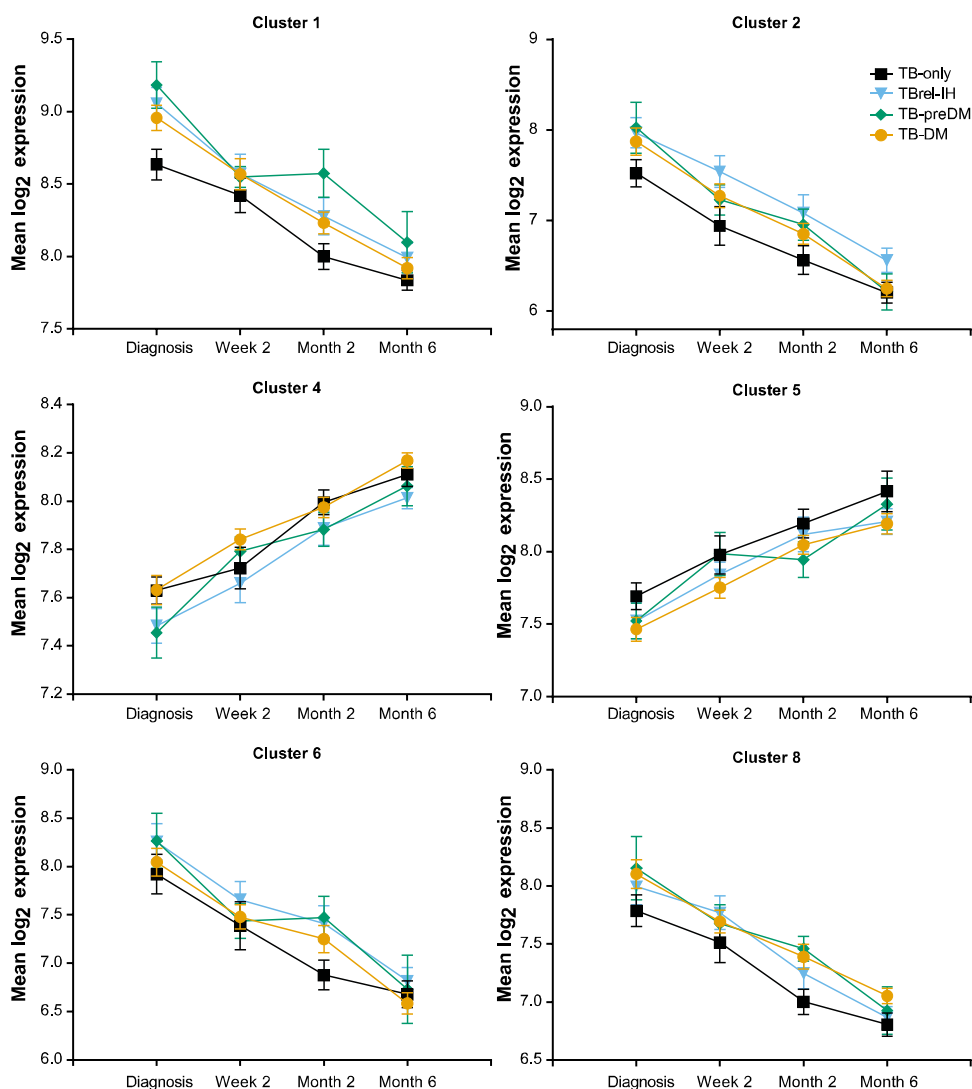


FIGURE 3 | Gene expression through treatment in TB patients with Pre-diabetes or TB-related intermediate hyperglycaemia, relative to TB-DM and TB-only patients. The expression of genes in the Core 102 genelist (Supplementary Table S7) was summed for those genes within each MaSigPro gene cluster (Figure 2) for individual patients (log₂ scale). Only MaSigPro clusters with > 3 genes in the core gene list are shown. Points show the mean $\pm$ SEM for each of the four clinical groups at each timepoint.

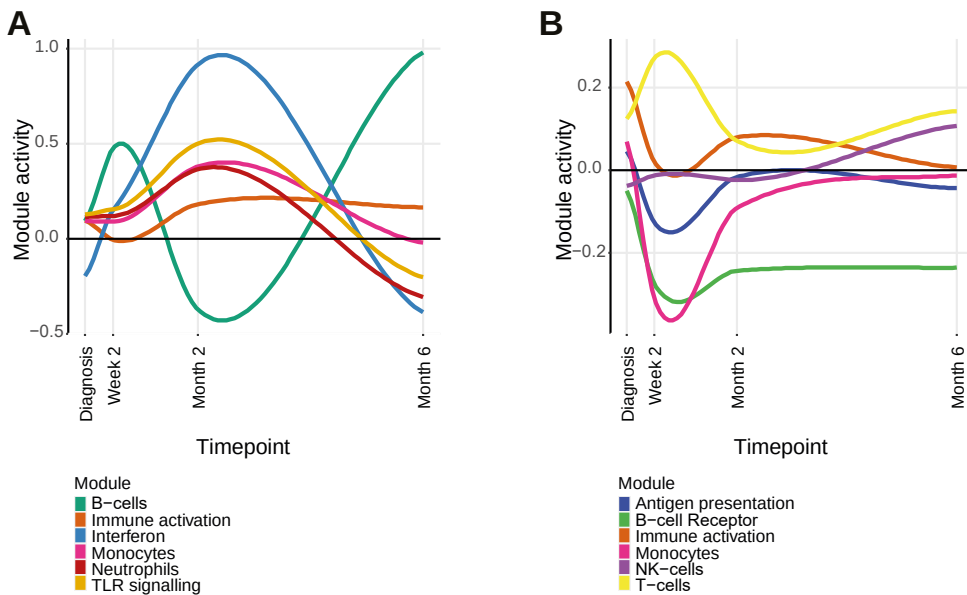


FIGURE 4 | Modular activity of the most significant modules in TB-DM relative to TB-only in A) South Africa and B) Indonesia. Modular analysis was performed between TB-DM and TB-only patients and the most statistically significant were chosen (p -value < 0.05). Modular activity calculated by summing the expression of genes within a module and dividing by the number of genes within that module.

through treatment, identified by Linear Mixed Models, showed some consistency across TB groups, with the South African cohort exhibiting downregulation of *GBP5*, *GBP1*, and *IFITM3* (Figure 6B) and the Indonesian cohort showing downregulation of *GBP5* and *IFITM3* and upregulation of *GNLY* (Supplementary Figure 6B) from diagnosis to 6 months. Importantly, the number of upregulated DEGs in response to TB treatment increased with glycaemic index in both cohorts (South Africa: TB-only:6 DEGs, TB-preDM:10 DEGs, TBrel-IH:12 DEGs, TB-DM:14 DEGs; Indonesia: TB-only: 9 DEGs, TBrel-IH:13 DEGs, TB-DM:22 DEGs). Notably, no DEGs were detected between 6 and 12 months in the South African cohort, except for *GBP5* ($p < 1e-10$) in patients with TBrel-IH (Supplementary Figure S7).

Ingenuity Pathway Analysis showed the majority of treatment-response DEGs in TB-only and TB-preDM were Interferon-signaling genes (ISGs) (Figure 6C, Supplementary Figure S6C). In contrast, in TBrel-IH and TB-DM patients, although downregulation of ISGs through treatment was observed, the major change was upregulation of genes associated with adaptive immunity (T-cell subset markers, Th1-associated genes, Treg-associated genes, cytotoxicity markers). Overall, the dcRT-MLPA confirmed that although TB-associated gene profiles show similar pattern and rate of change in TB patients and TB-DM, the magnitude is different.

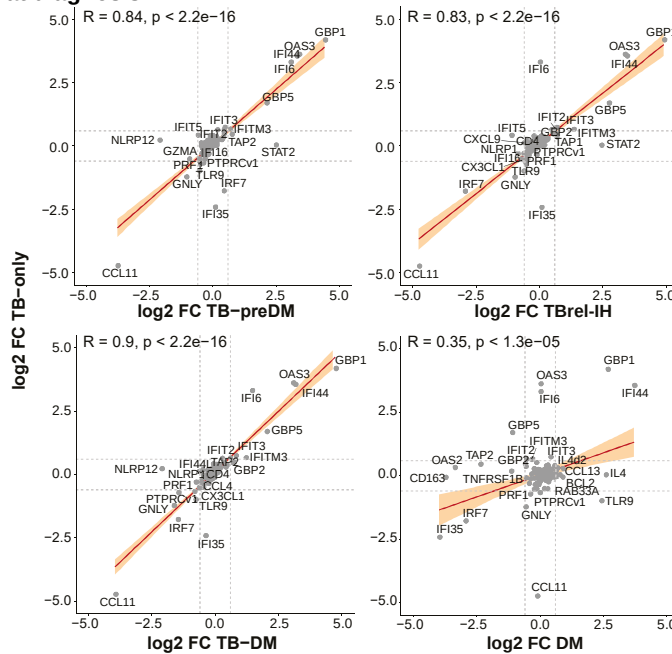
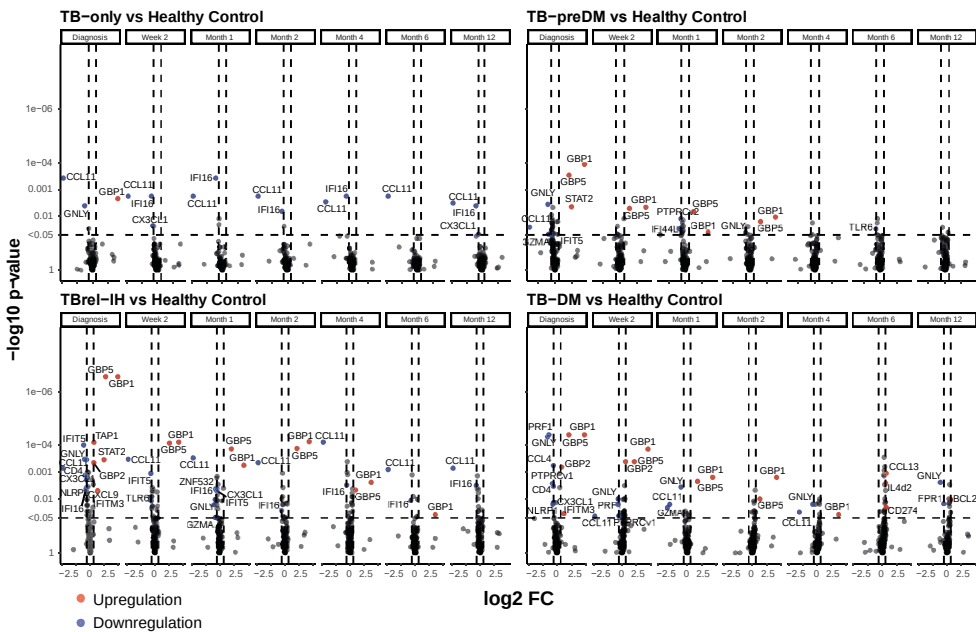
A Pearson at diagnosis**B Volcano plots: Study groups versus healthy controls - South Africa**

FIGURE 5 | Gene expression profiles in TBrel-IH and TB-DM are not completely normalised to healthy control profiles at the end of TB treatment. A) Scatter plots representing Pearson correlations between expression of all genes in targeted dcRT-MLPA panel in TB patients relative to healthy controls (y-axes) versus the other study groups relative to healthy controls (x-axes), plotted

as log2 FC. Red line corresponds to line of best fit and shaded bands indicate confidence intervals. Genes regulated log2 FC <-0.6 or > 0.6 are annotated. B) Differential Expression Analysis was performed on GAPDH-normalized log2-transformed targeted gene expression data of the South African cohort. Volcano plots representing DEGs at diagnosis and at different timepoints post TB treatment initiation of TB patients categorized based on their diabetes/glycaemia status compared to the healthy controls. The y-axis scales of all plots are harmonized per study group. P-values, -log10-transformed for better visualization, are plotted against log2 FC. Genes with $p < 0.05$ and log2 FC <-0.6 or > 0.6 were labelled as DEGs.

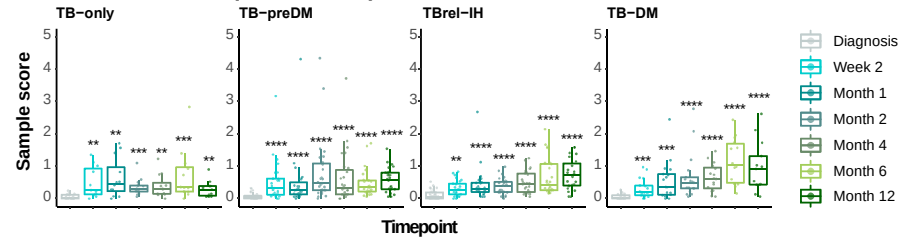
Identification of a signature for TB treatment-response

As TB transcriptomic signatures were altered in people with DM or IH, we identified signatures with the highest classifying power to discriminate between patients at diagnosis and end of TB treatment irrespective of diabetes/glycaemia by pooling all TB patients, using logistic regression with lasso regularisation. Initially, signatures were developed in the South African and Indonesian cohorts separately (Table 2; Supplementary Table S12). The classifying capability of each signature against the training (AUC range: 0.73 – 1.0) and validation (AUC range: 0.69 – 0.92) cohorts for each clinical group was reasonably good (Figure 7A&B, Supplementary Figure 8A&B). To improve the classification performance and reduce cohort dependency, the datasets of both cohorts were pooled, and a combined two cohort 15-gene signature developed. This showed enhanced classification performance across the cohorts, with ROC analysis showing AUCs of 0.88 for TB-only, 0.96 for TBrel-IH and 0.85 for TB-DM, with excellent classification retained in individual cohorts (Figure 7C; Supplementary Figure 8C). The kinetic profiles of 6 representative genes are shown in Supplementary Figure S9.

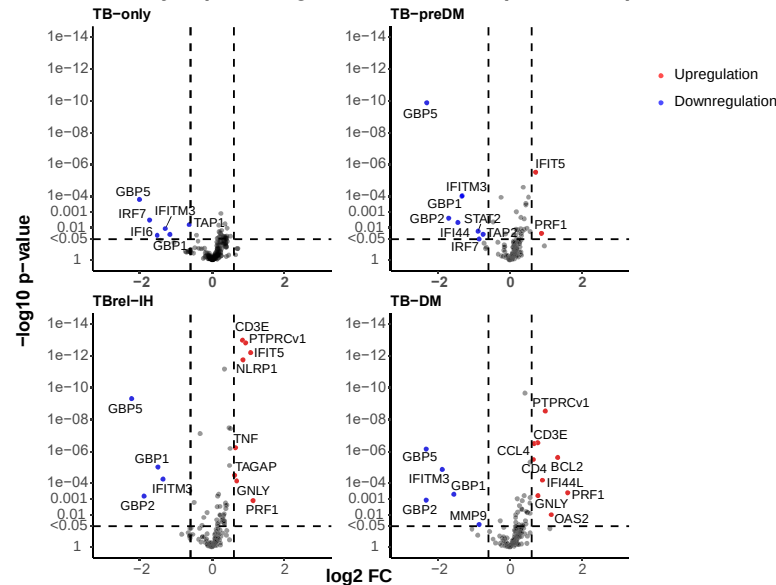
Discussion

In this longitudinal analysis of blood transcriptomes, excessive gene expression perturbation previously described at TB diagnosis [13, 25] continued throughout six months of TB treatment in pulmonary TB patients with diabetes co-morbidity. However, qualitatively and kinetically similar changes occurred in patients with or without diabetes, suggesting prolonged TB treatment might be sufficient to restore normal transcriptomes. TB patients with either pre-diabetes or TB-related IH also exhibited greater magnitudes of gene expression perturbation throughout treatment, similar to patients with diagnosed diabetes. The overall consistency in change of gene expression through treatment, irrespective of glycaemic index, enabled derivation of accurate predictive models of TB treatment response, which could be used effectively in populations with or without diabetes.

A MDP South Africa (0-12 months)



B Differentially expressed genes South Africa (0-6 months)



C IPA network analysis South Africa (0-6 months)

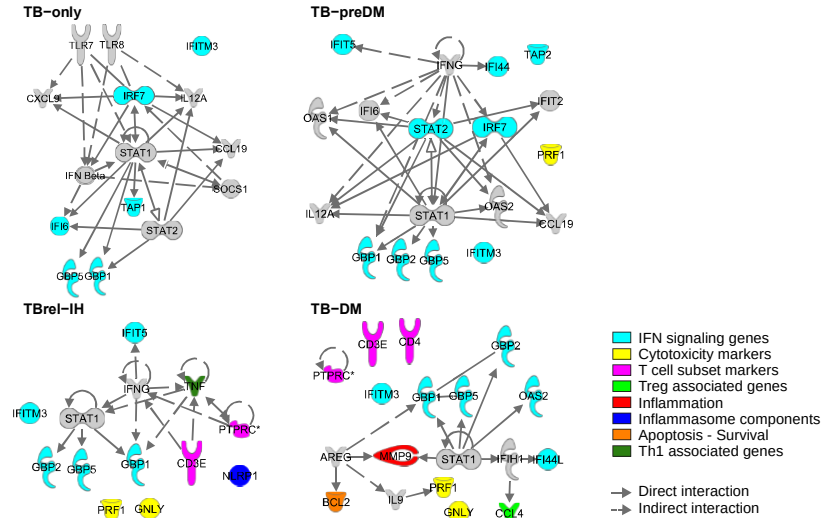


FIGURE 6 | TB treatment response in TB patients is dependent on diabetes/glycaemia status. MDP and Differential Expression Analyses were performed on GAPDH-normalized log2-transformed targeted gene expression data of the South African cohort. (A) MDP analyses of the different study groups showing the impact of TB treatment on the overall gene perturbation over time. Samples of patients at diagnosis were used as baseline controls. (B) Volcano plots representing DEGs regulated during TB treatment of TB patients categorized based on their diabetes/glycaemia status. The y-axis scales of all plots are harmonized per study group. P-values, $-\log_{10}$ -transformed for better visualization, are plotted against log2 FC. Genes with $p < 0.05$ and log2 FC < -0.6 or > 0.6 were labelled as DEGs. (C) IPA interactive network analyses of DEGs regulated during TB treatment. The various shapes of the nodes represent the functional classes of the gene products. Gene modules are indicated by distinctive colours.

TABLE 2 | Gene expression signature predicting month 6 versus diagnosis, obtained by pooling the study groups and cohorts (South Africa + Indonesia).

Gene	Module	Coefficient
Intercept		-14.99967
BLR1	G protein-coupled receptors	0.07841
CCL13	Chemokines	0.40415
CCL4	Treg associated genes	0.66015
CD19	Immune cell subset markers - B cells	0.2371
CD3E	T cell subset markers	0.35071
CD4	T cell subset markers	0.00263
FCGR1A	IFN signaling genes	-0.48224
FPR1	Myeloid-associated genes	-0.1725
GBP5	IFN signaling genes	-0.16957
IFIT5	IFN signaling genes	0.0172
NLRP1	Inflammasome components	0.3498
PTPRCv1	T cell subset markers	0.6625
TAP1	IFN signaling genes	-0.51107
TNF	Th1 associated genes	0.06539
ZNF532	Transcriptional regulators/activators	0.06389

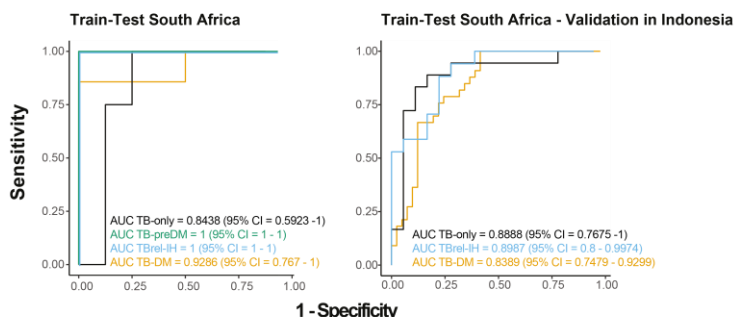
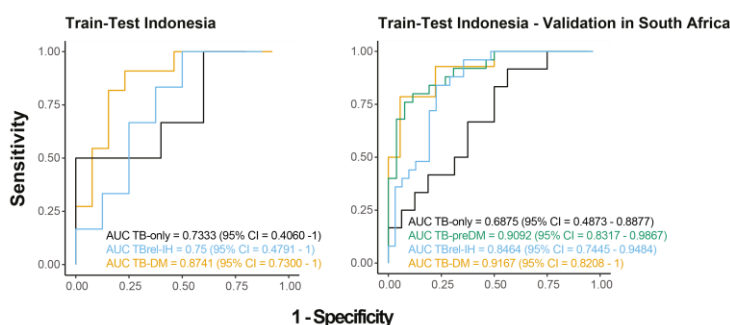
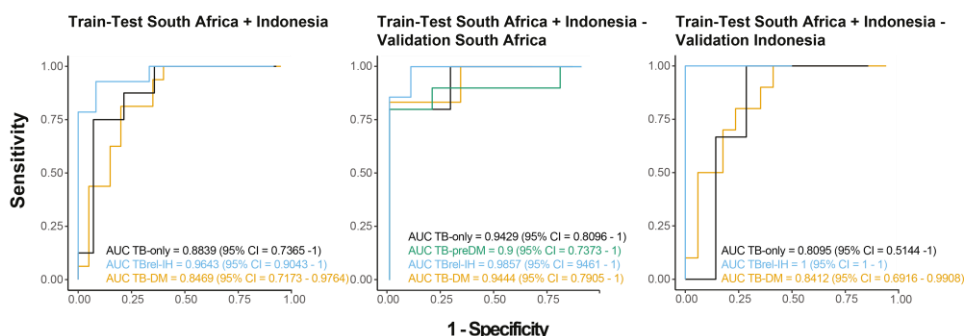
A South Africa**B Indonesia****C South Africa and Indonesia**

FIGURE 7 | Identification of common host biomarker signatures associated with TB treatment response irrespective of population heterogeneity and diabetes/glycaemia severity. South African, Indonesian, or pooled cohort transcriptomic datasets of TB patients independent of their diabetes/glycaemia status were used to train the models. Receiver Operating Characteristic (ROC) curves (Sensitivity plotted against 1-Specificity) and Area Under the Curve (AUC) with 95% Confidence Intervals (CI) show the classifying performance of the trained models. (A) The model trained on 70% of the South African dataset was tested in the remaining 30% of the South African dataset split into the different TB study groups (left panel) and validated using the complete dataset of the Indonesian cohort split into the different TB study groups (right panel). (B) The model trained on 70% of the Indonesian dataset was tested in the remaining 30% of the Indonesian dataset split into the different TB study groups (left panel) and validated using the complete dataset of the South

African cohort split into the different TB study groups (right panel). (C) The model trained on 70% of the pooled (South African and Indonesian) dataset was tested in the remaining 30% of the pooled dataset split into the different TB study groups that both cohorts have in common (left panel) and validated using the complete dataset of the South African cohort split into the different TB study groups (middle panel) or the complete dataset of the Indonesian cohort split into the different TB study groups (right panel).

Diabetes has a negative effect on TB treatment outcomes [5, 6], for unclear reasons. One explanation could be a qualitatively different immune response in diabetes, leaving people persistently susceptible to bacterial replication and disease reactivation. An alternative explanation is that excessive inflammation and immune activation at diagnosis in TB-DM means patients require longer or, more likely, different treatment to reach the same endpoint as people with uncomplicated TB, so that they are not left susceptible to TB recurrence. Our data support the latter model, as all gene clusters differentially expressed between clinical groups exhibited similar changes, but of different magnitude. Bronchial spread often persists beyond treatment initiation, with new or expanding cavities appearing on PET-CT scans 4 weeks into treatment in one-fifth of pulmonary TB patients [32]. Plausibly, increased ongoing bacterial spread in patients with diabetes co-morbidity causes persistent pro-inflammatory responses: the peripheral transcriptome correlates with lung inflammatory activity in TB patients [33]. Restoration of normal transcriptomes, and presumably improved lung resolution, could potentially also be achieved by coadministration of host-directed therapy alongside standard treatment. Therapy which dampens pro-inflammatory responses, such as corticosteroids or matrix metalloproteinase inhibitors [34], would have added benefit by reducing lung damage, which often persists after microbiological cure [35]. Anti-hyperglycaemic therapy, such as metformin, leads to more balanced, less inflammatory responses to *M.tuberculosis* [36], and has been suggested as adjunctive therapy for TB, particularly in patients with diabetes [37]. Our transcriptomic data suggest that patients with either pre-diabetes or TB-related IH would also benefit from prolonged or adjunctive host-directed therapy, in alignment with observed worse TB treatment outcomes in people with transient hyperglycaemia [38].

The ability to monitor TB treatment and predict outcome would be beneficial for clinical management. We show that transcriptomic models can be derived from host blood which reflect TB treatment-response irrespective of glycaemia. The best models include genes involved in interferon signalling, known to be suppressed at TB diagnosis in TB-DM patients [25], which we found were enhanced mid-way through treatment but did eventually resolve by 6 months. People protected against TB development display balanced prostaglandin 2 and lipoxin expression in lungs, preventing TB disease

progression following infection [39]. Drugs which target 5-lipoxygenase restrict lung pathology and reduce bacterial replication in murine models, by lowering the type 1 interferon response; the increases through treatment in the TB-DM cohort may relate to sustained infection and accompanying inflammation. In TB-DM patients the inflammation-related genes resolved more linearly through TB treatment, but remained elevated to the end of TB treatment, persisting until 12 months post-diagnosis in the South African cohort. In future studies, it would be important to test whether prolonged treatment with standard therapy impacts blood transcriptomes beyond the 6 month time point. Increased doses of anti-TB drugs might also lead to better treatment outcomes in TB-DM. In a companion paper (van Doorn *et al*, [40] unpublished), transcriptomic signatures indicative of treatment outcome have been derived that can be used in patients with either DM or IH. Together, these papers show that signatures related to poor TB outcome are distinct from the excessive and prolonged inflammation observed in TB-DM.

These findings further illustrate how comorbidity with diabetes affects the host response to *M.tuberculosis* infection, and how a better understanding of these interactions could be exploited to reduce poor TB treatment outcomes associated with TB and diabetes comorbidity.

Funding

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Conflicts of Interest

GW has patents about methods of tuberculosis diagnosis and tuberculosis biomarkers which are unrelated to the current study. No other authors have any declared conflicts of interest.

Acknowledgments

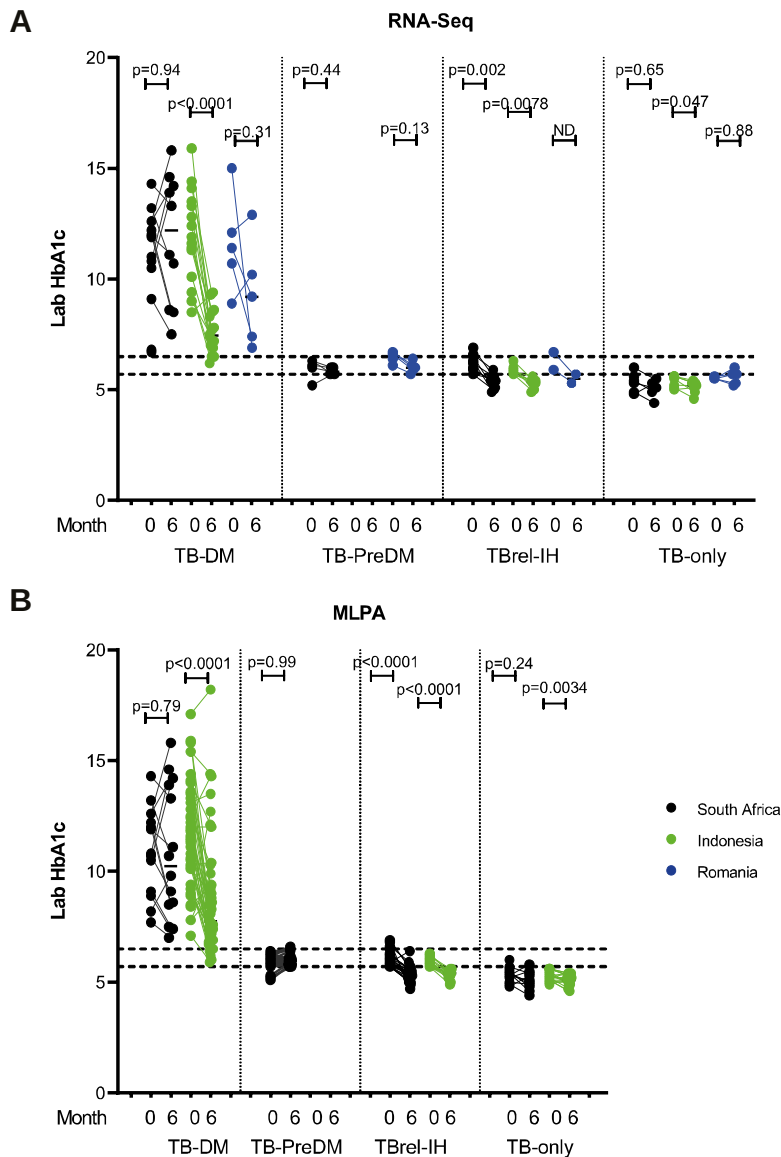
We are grateful to all the study participants for blood and data donations. We acknowledge Bahram Sanjabi, Desiree Brandenburg-Weening, and Pieter van der Vlies for assistance with the RNA-Seq, Evelien Temminck for providing technical assistance with dcRT-MLPA experiments, and Erni Durdevic for providing statistical and machine learning advice.

References

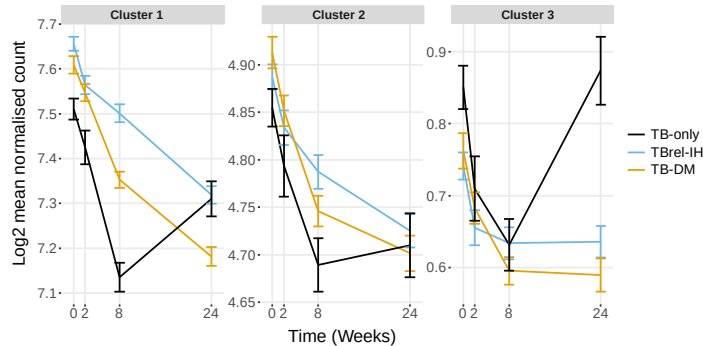
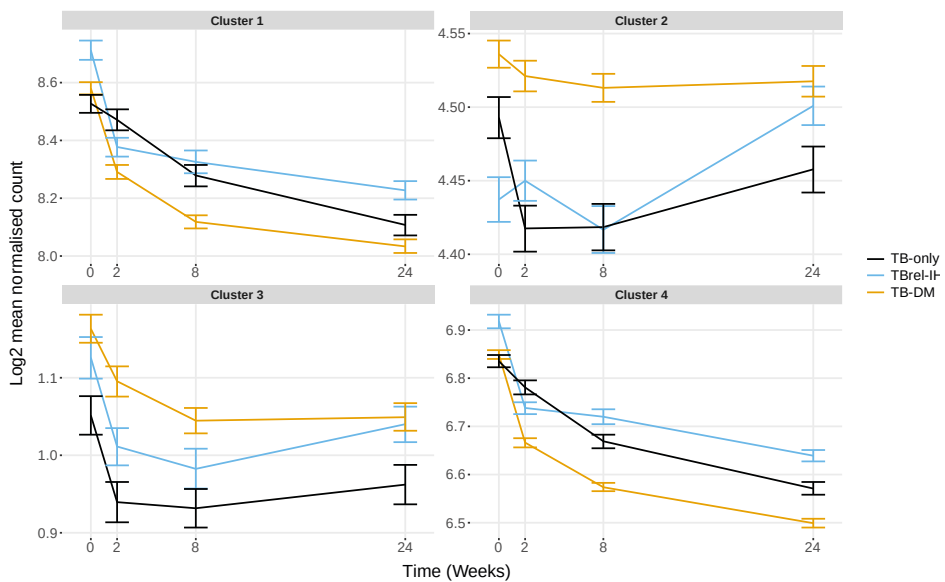
1. Koesoemadinata RC, McAllister SM, Soetedjo NNM, et al. Latent TB infection and pulmonary TB disease among patients with diabetes mellitus in Bandung, Indonesia. *Trans R Soc Trop Med Hyg* 2017; 111(2): 81-9.
2. Al-Rifai RH, Pearson F, Critchley JA, Abu-Raddad LJ. Association between diabetes mellitus and active tuberculosis: A systematic review and meta-analysis. *PLoS One* 2017; 12(11):e0187967.
3. Jeon CY, Murray MB. Diabetes mellitus increases the risk of active tuberculosis: a systematic review of 13 observational studies. *PLoS Med* 2008; 5(7): e152.
4. Noubiap JJ, Nansseu JR, Nyaga UF, et al. Global prevalence of diabetes in active tuberculosis: a systematic review and meta-analysis of data from 2.3 million patients with tuberculosis. *Lancet Glob Health* 2019; 7(4): e448-e60.
5. Baker MA, Harries AD, Jeon CY, et al. The impact of diabetes on tuberculosis treatment outcomes: a systematic review. *BMC Med* 2011; 9: 81.
6. Huangfu P, Ugarte-Gil C, Golub J, Pearson F, Critchley J. The effects of diabetes on tuberculosis treatment outcomes: an updated systematic review and meta-analysis. *Int J Tuberc Lung Dis* 2019; 23(7): 783-96.
7. International Diabetes Federation. *IDF Diabetes Atlas Ninth Edition*, 2019.
8. American Diabetes Association. Diagnosis and classification of diabetes mellitus. *Diabetes Care* 2010; 33 Suppl 1: S62-9.
9. Dungan KM, Braithwaite SS, Preiser JC. Stress hyperglycaemia. *Lancet* 2009; 373(9677):1798-807.
10. Boillat-Blanco N, Ramaiya KL, Mganga M, et al. Transient Hyperglycemia in Patients With Tuberculosis in Tanzania: Implications for Diabetes Screening Algorithms. *J Infect Dis* 2016;213(7): 1163-72.
11. Yoon YS, Jung JW, Jeon EJ, et al. The effect of diabetes control status on treatment response in pulmonary tuberculosis: a prospective study. *Thorax* 2017; 72(3): 263-70.
12. Ronacher K, Joosten SA, van Crevel R, Dockrell HM, Walzl G, Ottenhoff TH. Acquired immunodeficiencies and tuberculosis: focus on HIV/AIDS and diabetes mellitus. *Immunol Rev* 2015; 264(1): 121-37.
13. Prada-Medina CA, Fukutani KF, Pavan Kumar N, et al. Systems Immunology of Diabetes-Tuberculosis Comorbidity Reveals Signatures of Disease Complications. *Sci Rep* 2017; 7(1):1999.
14. Ronacher K, Chegou NN, Kleynhans L, et al. Distinct serum biosignatures are associated with different tuberculosis treatment outcomes. *Tuberculosis (Edinb)* 2019; 118: 101859.
15. Kumar NP, Banurekha VV, Nair D, et al. Coincident pre-diabetes is associated with dysregulated cytokine responses in pulmonary tuberculosis. *PLoS One* 2014; 9(11): e112108.
16. Kumar NP, Fukutani KF, Shruthi BS, et al. Persistent inflammation during anti-tuberculosis treatment with diabetes comorbidity. *Elife* 2019; 8.
17. Kumar NP, Moideen K, Sivakumar S, et al. Modulation of dendritic cell and monocyte subsets in tuberculosis-diabetes co-morbidity upon standard tuberculosis treatment. *Tuberculosis (Edinb)* 2016; 101: 191-200.
18. Kumar NP, Moideen K, Viswanathan V, Kornfeld H, Babu S. Effect of standard tuberculosis treatment on naive, memory and regulatory T-cell homeostasis in tuberculosis-diabetes comorbidity. *Immunology* 2016; 149(1): 87-97.
19. Berry MP, Graham CM, McNab FW, et al. An interferon-inducible neutrophil-driven blood transcriptional signature in human tuberculosis. *Nature* 2010; 466(7309): 973-7.
20. Cliff JM, Lee JS, Constantinou N, et al. Distinct phases of blood gene expression pattern through tuberculosis treatment reflect modulation of the humoral immune response. *J Infect Dis* 2013; 207(1): 18-29.
21. Kaforou M, Wright VJ, Oni T, et al. Detection of tuberculosis in HIV-infected and -uninfected

- African adults using whole blood RNA expression signatures: a case-control study. *PLoS Med* 2013; 10(10): e1001538.
22. Ottenhoff TH, Dass RH, Yang N, et al. Genome-wide expression profiling identifies type 1 interferon response pathways in active tuberculosis. *PLoS One* 2012; 7(9): e45839.
 23. Bloom CI, Graham CM, Berry MP, et al. Detectable changes in the blood transcriptome are present after two weeks of antituberculosis therapy. *PLoS One* 2012; 7(10): e46191.
 24. Thompson EG, Du Y, Malherbe ST, et al. Host blood RNA signatures predict the outcome of tuberculosis treatment. *Tuberculosis* 2017; 107: 48-58.
 25. Eckold C, Kumar V, Weiner J, et al. Impact of Intermediate Hyperglycemia and Diabetes on Immune Dysfunction in Tuberculosis. *Clin Infect Dis* 2021; 72(1): 69-78.
 26. Ugarte-Gil C, Alisjahbana B, Ronacher K, et al. Diabetes Mellitus Among Pulmonary Tuberculosis Patients From 4 Tuberculosis-endemic Countries: The TANDEM Study. *Clin Infect Dis* 2020; 70(5): 780-8.
 27. Ruslami R, Koesoemadinata RC, Soetedjo NNM, et al. The effect of a structured clinical algorithm on glycemic control in patients with combined tuberculosis and diabetes in Indonesia: A randomized trial. *Diabetes research and clinical practice* 2021; 173: 108701.
 28. Grint D, Alisjahbana B, Ugarte-Gil C, et al. Accuracy of diabetes screening methods used for people with tuberculosis, Indonesia, Peru, Romania, South Africa. *Bull World Health Organ* 2018; 96(11): 738-49.
 29. Nueda MJ, Tarazona S, Conesa A. Next maSigPro: updating maSigPro Bioconductor package for RNA-seq time series. *Bioinformatics* 2014; 30(18): 2598-602.
 30. Joosten SA, Goeman JJ, Sutherland JS, et al. Identification of biomarkers for tuberculosis disease using a novel dual-color RT-MLPA assay. *Genes Immun* 2012; 13(1): 71-82.
 31. Dennis G, Jr., Sherman BT, Hosack DA, et al. DAVID: Database for Annotation, Visualization, and Integrated Discovery. *Genome Biol* 2003; 4(5): P3.
 32. Chen RY, Yu X, Smith B, et al. Radiological and functional evidence of the bronchial spread of tuberculosis: an observational analysis. *The Lancet Microbe* 2021; 2(10): e518-e26.
 33. Odia T, Malherbe ST, Meier S, et al. The Peripheral Blood Transcriptome Is Correlated With PET Measures of Lung Inflammation During Successful Tuberculosis Treatment. *Frontiers in immunology* 2020; 11: 596173.
 34. Young C, Walzl G, Du Plessis N. Therapeutic host-directed strategies to improve outcome in tuberculosis. *Mucosal Immunology* 2020; 13(2): 190-204.
 35. Krug S, Parveen S, Bishai WR. Host-Directed Therapies: Modulating Inflammation to Treat Tuberculosis. *Frontiers in immunology* 2021; 12: 660916.
 36. Lachmandas E, Eckold C, Bohme J, et al. Metformin Alters Human Host Responses to *Mycobacterium tuberculosis* in Healthy Subjects. *J Infect Dis* 2019; 220(1): 139-50.
 37. Singhal A, Jie L, Kumar P, et al. Metformin as adjunct antituberculosis therapy. *Science translational medicine* 2014; 6(263): 263ra159.
 38. Liu Q, You N, Pan H, et al. Glycemic Trajectories and Treatment Outcomes of Patients with Newly Diagnosed Tuberculosis: A Prospective Study in Eastern China. *Am J Respir Crit Care Med* 2021; 204(3): 347-56.
 39. Mayer-Barber KD, Andrade BB, Oland SD, et al. Host-directed therapy of tuberculosis based on interleukin-1 and type I interferon crosstalk. *Nature* 2014; 511(7507): 99-103.
 40. van Doorn CLR, Eckold C, Ronacher K, et al. Transcriptional profiles predict treatment outcome in patients with tuberculosis and diabetes at diagnosis and at two weeks after initiation of anti-tuberculosis treatment. *MedRxiv* 2022; (MEDRXIV/2022/269796).

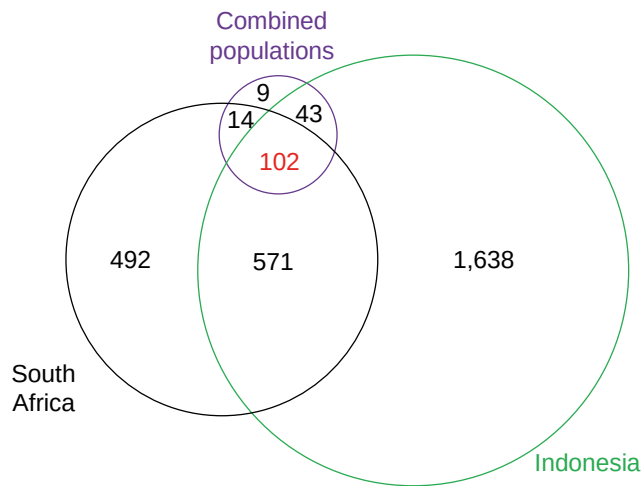
Supplementary Figures



SUPPLEMENTARY FIGURE S1 | Changes in HbA1c in pulmonary TB patients through TB drug treatment in people with concomitant diabetes, pre-diabetes or TB-related intermediate hyperglycaemia. Laboratory HbA1c recorded at month 0, prior to initiation of treatment, and at the end of treatment at month 6 in patients included in A) RNA-Seq analysis and B) IMLPA analysis, for cohorts from South Africa (Black), Indonesia (Green) and Romania (Blue). The dotted lines show the threshold for diagnosis of diabetes (6.5%) and pre-diabetes (5.7%) [8]. Timepoints in each cohort were compared by Wilcoxon signed rank test.

A South Africa**B Indonesia**

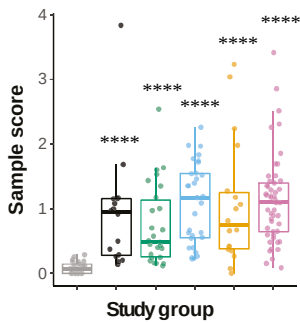
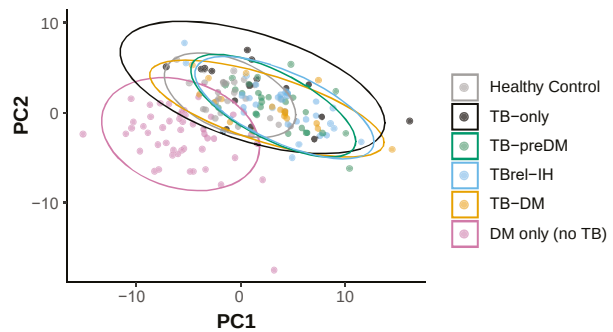
SUPPLEMENTARY FIGURE S2 | MaSigPro analysis of change in gene expression through TB treatment in blood samples from patients in A) South Africa and B) Indonesia. MaSigPro identified genes that behaved similarly through time and between patient groups using hierarchical clustering. Results are shown for log-transformed normalised transcript counts for the TB-only, TB-DM and the combined TB-PreDM and TBrel-IH groups. Bars show mean ± 1 SEM. Data were filtered to remove lowly abundant transcripts prior to analysis. NB: There were insufficient study participants to conduct an analysis separately for the Romanian population.



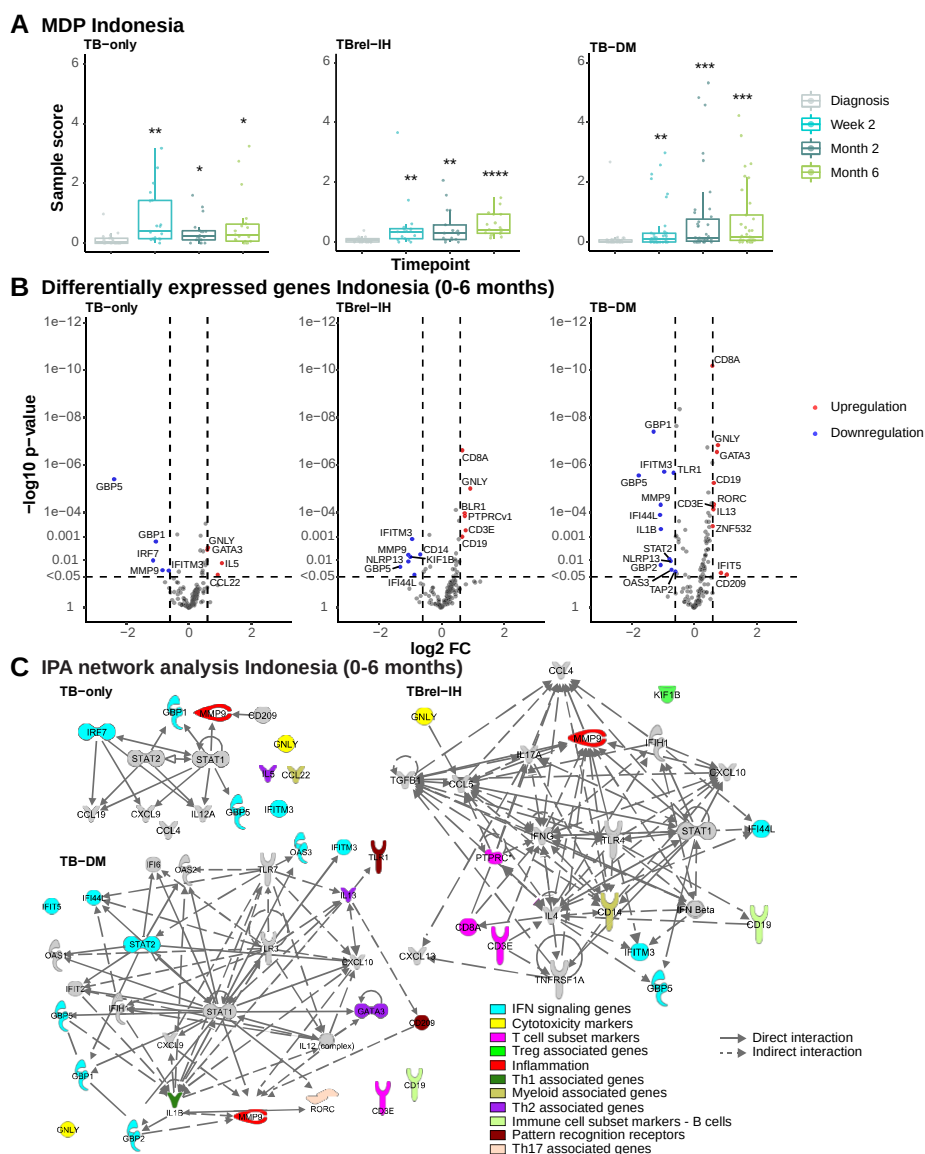
SUPPLEMENTARY FIGURE S3 | Comparison of results from MaSigPro analyses for individual and combined populations. Genes which were identified as behaving differently between TB-DM, TBrel-IH/TB-PreDM or TB-only in South Africa (Black), Indonesia (Green) or combined populations from Romania, South Africa and Indonesia (Purple) in MaSigPro analyses (Figures 2, S2; Supplementary Tables S4, S5, S6) were compared. The Venn Diagram shows the number of identified genes which overlap between analyses



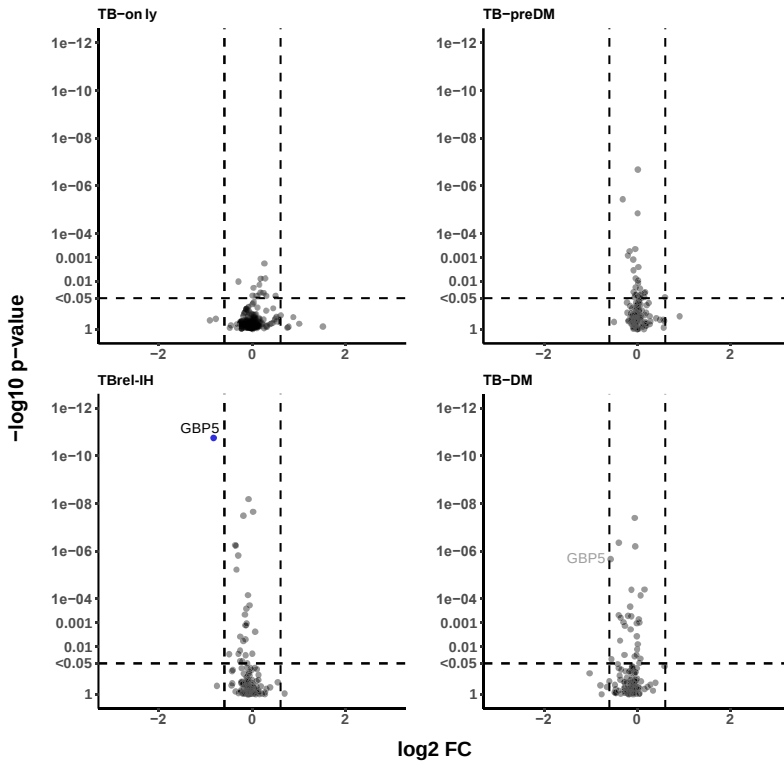
SUPPLEMENTARY FIGURE S4 | G:profiler analysis of 102 gene MSP core gene list, identifying top Gene Ontology (GO) molecular function (MF), biological process (BP) and cellular component (CC) as well as biological pathways in KEGG and Reactome databases.

A MDP at diagnosis**B PLS-DA at diagnosis**

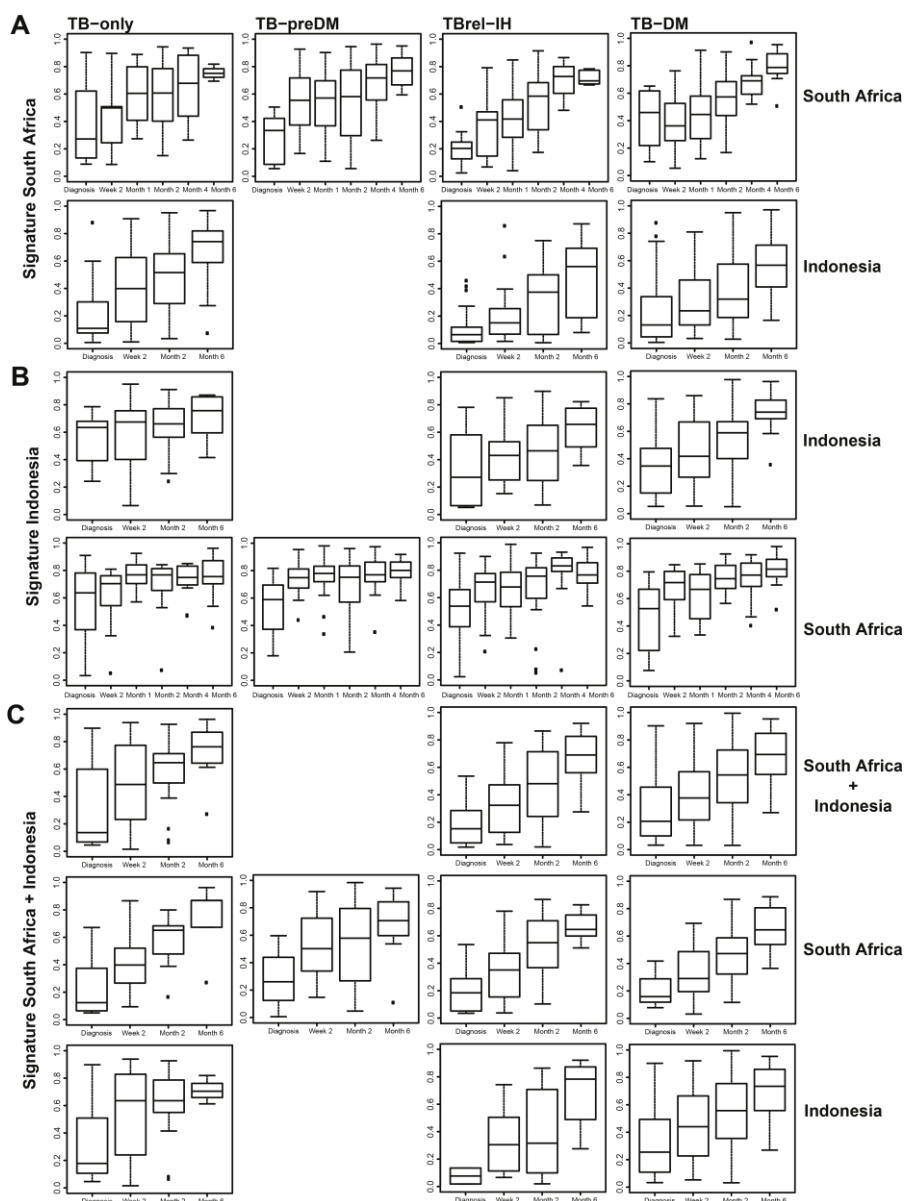
SUPPLEMENTARY FIGURE S5 | Gene expression profiles in TB patients is dominant over the gene perturbation caused by DM. Molecular Degree of Perturbation (MDP) and Partial Least Squares – Discriminant Analysis (PLS-DA) were performed on GAPDH-normalized log2-transformed targeted gene expression data of the South African cohort. (A) MDP analysis of the different study groups showing the impact of TB and DM on the overall gene perturbation at diagnosis. Samples of healthy individuals were used as controls. (B) PLS-DA analysis of the different study groups at diagnosis. Samples of healthy individuals were used as controls.



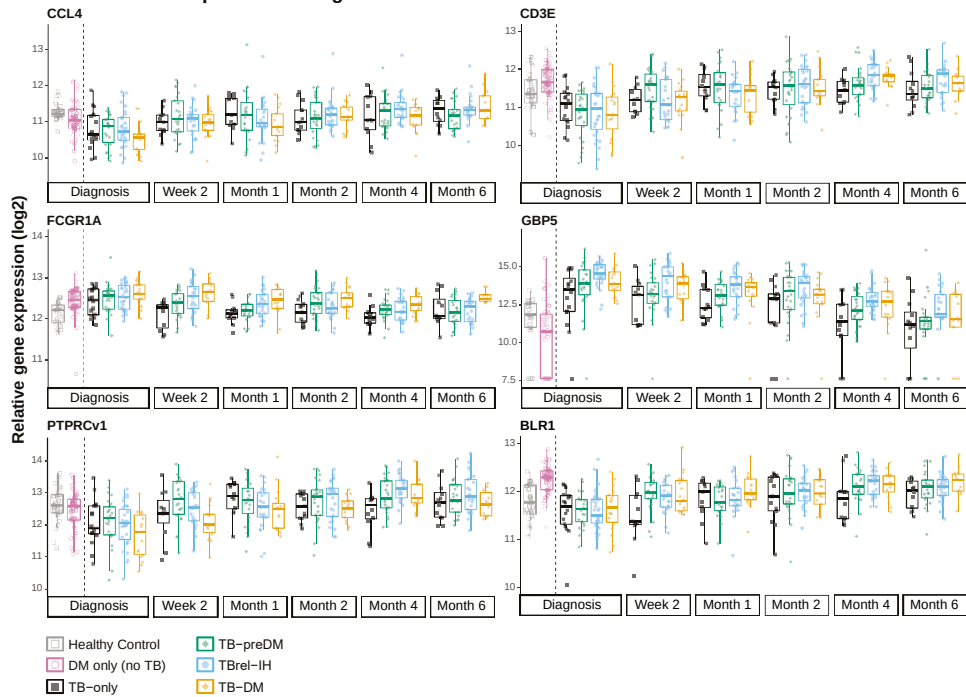
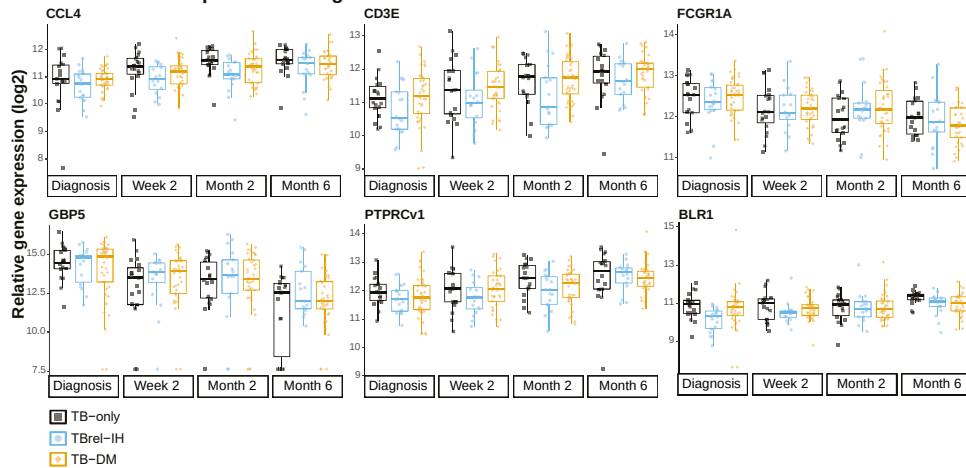
SUPPLEMENTARY FIGURE S6 | TB treatment response in TB patients is dependent on diabetes/glycaemia status in the Indonesian cohort. MDP and Differential Expression Analyses were performed on GAPDH-normalized log₂-transformed targeted gene expression data of the Indonesian cohort. (A) MDP analyses of the different study groups showing the impact of TB treatment on the overall gene perturbation over time. Samples of patients at diagnosis were used as baseline controls. (B) Volcano plots representing DEGs regulated during treatment of TB patients categorized based on their diabetes/glycaemia status. The y-axis scales of all plots are harmonized per study group. -log₁₀-transformed p-values are plotted against log₂ FC. Genes with p < 0.05 and log₂ FC < -0.6 or > 0.6 were labelled as DEGs. (C) IPA interactive network analyses of DEGs regulated during treatment. The various shapes of the nodes represent the functional classes of the gene products. Gene modules are indicated by distinctive colors.

Differentially expressed genes South Africa after anti-TB treatment (6-12 months)

SUPPLEMENTARY FIGURE S7 | Transcriptional response after anti-TB treatment in the South African cohort. Volcano plots representing DEGs regulated after TB treatment (6-12 months) of TB patients categorized based on their diabetes/glycaemia status. The y-axis scales of all plots are harmonized per study group. P-values, $-\log_{10}$ -transformed for better visualization, are plotted against $\log_2 FC$. Genes with $p < 0.05$ and $\log_2 FC < -0.6$ or > 0.6 were labelled as DEGs.



SUPPLEMENTARY FIGURE S8 | Classifying capability of identified TB treatment response biomarker signatures. Predicted probability plots showing the accuracy of TB treatment response biomarker signatures identified in (A) South Africa or (B) Indonesia across timepoints in box-and-whiskers plots (5-95 percentiles) either in the cohort in which the Train Test Split (TTS) was performed or in the validation cohort, split into TB study groups based on their diabetes/glycaemia status. (C) Predicted probability plots showing the accuracy of the identified pooled (South Africa and Indonesia) biomarker signature across timepoints in box-and-whiskers plots (5-95 percentiles) either in the pooled cohort in which Train-Test Split (TTS) was performed (left panel) or the single validation cohorts (right panels), split into TB study groups based on their diabetes/glycaemia status.

A Scatter-and-box-plots of the signature - South Africa**B Scatter-and-box-plots of the signature - Indonesia**

SUPPLEMENTARY FIGURE S9 | The kinetic profiles of 6 representative genes of the 15-gene TB treatment response signature of the pooled cohort dataset. Expression kinetics of single genes in the (A) South African cohort or (B) Indonesian cohort at diagnosis and during TB treatment. Box plots depict log2-transformed median gene expression values and the inter quartile range (IQR), while the whiskers represent the data within the Q1-1.5xIQR and Q3+1.5xIQR interval. Outliers are reported as symbols.

Supplementary Tables

SUPPLEMENTARY TABLE S1 | List of target genes for dcfRT-MLPA.

Module	Gene
Immune cell subset markers - B cells NK cells	CD19
	NCAM1
T cell subset markers	CD3E
	CD4
	CD8A
	CCR7
	PTPRCv1
	PTPRCv2
	AIRE
	IL7R
Th1 associated / IFN signaling genes	CXCL10
Th1 associated genes	IFNG
	IL1B
	IL2
	IL15
	TBX21
	TNF
Th2 associated genes	GATA3
	IL4
	IL452
	IL5
	IL6
	IL10
	IL13
Th9 associated genes	IL9
Th17 associated genes	IL17A
	RORC
	IL22RA1
Treg associated genes	CCL4
	CTLA4
	FOXP3
	IL2RA
	LAG3
	TGFB1
	TNFRSF18
Cytotoxicity markers	GNLY
	GZMA
	GZMB
	PRF1
Apoptosis / Survival	CASP8
	BCL2
	FASLG
	FLCN1
	TNFRSF1A
	TNFRSF1B
Myeloid-associated genes	CD14
	CD163

	CCL2
	CCL3
	CCL5
	CCL22
	CXCL13
	IL12A
	IL12B
	IL23A
	FPR1
Chemokines	CCL11
	CCL13
	CCL19
	CXCL9
	CX3CL1
Pattern recognition receptors	CD209
	CLEC7A
	MRC1
	MRC2
	NOD1
	NOD2
	TLR1
	TLR2
	TLR3
	TLR4
	TLR5
	TLR6
	TLR7
	TLR8
	TLR9
	TLR10
Inflammasome components	NLR4
	NLRP1
	NLRP2
	NLRP3
	NLRP4
	NLRP6
	NLRP7
	NLRP10
	NLRP11
	NLRP12
	NLRP13
IFN signaling genes	CD274
	FCGR1A
	GBP1
	GBP2
	GBP5
	IFI6
	IFI16
	IFI35
	IFI44
	IFI44L

	IFIH1 IFIT2 IFIT3 IFIT5 IFITM1/3 INDO IRF7 OAS1 OAS2 OAS3 SOCS1 STAT1 STAT2 TAP1 TAP2
Inflammation	DSE MMP9 SPP1 TIMP2 TNIP1
Cell growth / Proliferation	BMP6 TGFB2 AREG EGF VEGF
Cell activation	HCK LYN SLAMF7
Small GTPases / (Rho) GTPase activating proteins	ASAP1 RAB13 RAB24 RAB33A TAGAP TBC1D7
Anti-microbial activity	BPI LTF
E3 ubiquitin protein ligases	NEDD4L
Scavenger receptors	MARCO
G protein-coupled receptors	BLR1
Transcriptional regulators/activators	CAMTA1 TWIST1 ZNF331 ZNF532
Intracellular transport	SEC14L1 KIF1B
Mitochondrial Stress / Proteasome	HPRT
Housekeeping	ABR B2M GAPDH GUSB

SUPPLEMENTARY TABLE S2 | Demographics of study participants in the three locations in RNA-Seq and MLPA study arms.

Characteristic	Country	TB-DM		TB-PreDM		TBrel-IH		TB-only		DM-only	HC
		RNA-Seq	MLPA	RNA-Seq	MLPA	RNA-Seq	MLPA	RNA-Seq	MLPA	MLPA	MLPA
Number of participants	S Africa	13	19	5	28	12	33	6	18	48	28
	Indonesia	16	48	0	0	8	19	7	22	-	-
	Romania	5	0	5	0	2	0	5	0	-	-
	All	34	67	10	28	22	52	18	40	48	28
Age in years, median (range)	S Africa	46 (27-57)	46 (27-57)	46 (43-56)	47 (24-68)	40 (25-55)	39 (22-62)	42 (32-56)	39 (19-56)	54 (19-69)	42 (30-70)
	Indonesia	50 (33-62)	50 (25-73)	-	-	44 (37-62)	43 (37-64)	48 (28-61)	45 (28-64)	-	-
	Romania	46 (22-60)	-	54 (44-75)	-	29 (22-36)	-	43 (30-64)	-	-	-
	All	48 (22-62)	50 (25-73)	54 (43-75)	47 (24-68)	40 (22-62)	40 (22-64)	45 (28-64)	43 (19-64)	54 (19-69)	42 (30-70)
Sex, % male (No. male/female)	S Africa	38 (5/8)	47 (9/10)	80 (4/1)	79 (22/6)	33 (4/8)	58 (19/14)	17 (1/5)	50 (9/9)	48 (23/25)	50 (14/14)
	Indonesia	50 (8/8)	54 (26/22)	-	-	50 (4/4)	58 (11/8)	71 (5/2)	59 (13/9)	-	-
	Romania	80 (4/1)	-	80 (4/1)	-	100 (2/0)	-	60 (3/2)	-	-	-
	All	50 (17/17)	52 (35/32)	80 (8/2)	79 (22/6)	45 (10/12)	58 (30/22)	50 (9/9)	55 (22/18)	48 (23/25)	50 (14/14)
HbA1c TB diagnosis, % median (range)	S Africa	11.9 (6.7-14.3)	10.7 (6.7-14.3)	6.1 (5.2-6.3)	6.0 (5.1-6.4)	6.0 (5.7-6.9)	5.9 (5.7-6.9)	5.4 (4.8-6.0)	5.4 (4.8-6.0)	10.5 (4.7-14.9)	5.3 (4.8-6.5)
	Indonesia	11.8 (8.5-15.9)	11.5 (7.1-17.1)	-	-	5.8 (5.7-6.3)	5.9 (5.7-6.3)	5.5 (5.0-5.6)	5.3 (4.9-5.6)	-	-
	Romania	11.4 (8.9-15.0)	-	6.6 (6.1-6.7)	-	6.3 (5.9-6.7)	-	5.5 (5.5-5.6)	-	-	-
	All	11.8 (6.7-15.9)	11.1 (6.7-17.1)	6.3 (5.2-6.7)	6.0 (5.1-6.4)	5.9 (5.7-6.9)	5.9 (5.7-6.9)	5.5 (4.8-6.0)	5.3 (4.8-6.0)	10.5 (4.7-14.9)	5.3 (4.8-6.5)
HbA1c at 6 months, % median (range)	S Africa	12.2 (7.5-15.8)	10.25 (7.0-15.8)	5.8 (5.7-6.0)	6.0 (5.7-6.6)	5.4 (4.9-5.9)	5.4 (4.7-6.4)	5.0 (4.4-5.3)	5.0 (4.4-5.8)	-	-
	Indonesia	7.5 (6.2-9.4)	7.7 (5.9-12.3)	-	-	5.4 (4.9-5.6)	5.4 (4.9-5.6)	5.2 (4.6-5.4)	5.1 (4.6-5.4)	-	-
	Romania	9.2 (6.9-12.9)	-	6.0 (5.7-6.4)	-	5.5 (5.3-5.7)	-	5.7 (5.2-6.0)	-	-	-
	All	8.5 (6.2-15.8)	8.3 (5.9-18.2)	6.0 (5.7-6.4)	6.0 (5.7-6.6)	5.4 (4.9-5.9)	5.4 (4.7-6.4)	5.3 (4.4-6.0)	5.1 (4.4-5.8)	-	-

SUPPLEMENTARY TABLE S3 | Diabetes diagnoses and treatment in TB-DM patients.

		S Africa		Indonesia		Romania		All	
		Pre-existing DM	New DM	Pre-existing DM	New DM	Pre-existing DM	New DM	Pre-existing DM	New DM
Number (%)		14 (74%)	5 (26%)	35 (73%)	13 (27%)	3 (60%)	2 (40%)	52 (72%)	20 (28%)
Medication, number	Insulin	2	0	9	3	3	0	14	3
	Metformin	6	0	19	8	0	0	25	8
	Other or combination	6	0	7	2	0	0	13	2

SUPPLEMENTARY TABLE S8 | Fixed effects (type III) longitudinal modelling of changes in gene expression in individual patients through time across 4 clinical groups (TB-DM, TB-preDM, TBrel-IH, TB-only).

Combined MaSigPro Cluster	Cluster 1	Cluster 2	Cluster 4	Cluster 5	Cluster 6	Cluster 8	Cluster 3	Cluster 7	Cluster 9
Number of genes in original Combined MSP	27	50	17	9	28	16	6	10	4
Number of genes in core list in combined	18	36	9	4	19	11	3	1	1
Clinical Group P-value	0.0217	0.0322	0.0439	0.244	0.2466	0.2283			
Time P-value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001			
Clinical Group x Time P-value	0.4996	0.8123	0.6796	0.8598	0.6779	0.8211			

Supplementary Table S4-S7 and S9-S11 can be downloaded at:

<https://www.medrxiv.org/content/10.1101/2022.02.07.22269422v1.supplementary-material>

SUPPLEMENTARY TABLE S12 | Signature month 6 versus diagnosis obtained by pooling the study groups in the South African cohort or the Indonesian cohort.**Signature South Africa**

Gene	Module	Coefficient
Intercept		-31.46231
BLR1	G protein-coupled receptors	0.52423
CCL4	Treg associated genes	0.74179
CD3E	T cell subset markers	0.64186
GBP5	IFN signaling genes	-0.39161
IFIT5	IFN signaling genes	0.07225
IFITM1/3	IFN signaling genes	-0.00135
NLRP1	Inflammasome components	0.033
PRF1	Cytotoxicity markers	0.0287
PTPRCv1	T cell subset markers	0.39243
SEC14L2	Intracellular transport	0.6171
TNF	Th1 associated genes	0.05957

Signature Indonesia

Gene	Module	Coefficient
Intercept		-1.15273
CCL13	Chemokines	0.34316
CCL4	Treg associated genes	0.10669
CD3E	T cell subset markers	0.1203
CD4	T cell subset markers	0.06428
FCGR1A	IFN signaling genes	-0.77562
FPR1	Myeloid-associated genes	-0.42241
GBP2	IFN signaling genes	-0.09952
GNLY	Cytotoxicity markers	0.34373
KIF1B	Intracellular transport	-0.11987
MMP9	Inflammation	-0.1168
RORC	Th17 associated genes	0.05677
TBX21	Th1 associated genes	0.75817

Supplementary Methods

RNASeq data analysis

RNA Samples processed for RNA-Seq analysis were quality-assessed by LabChip GX HiSens RNA system (PerkinElmer). Total RNA samples were processed using the poly-A tail Bioscientific NEXTflex-Rapid-Directional mRNA-seq method with the Caliper SciClone to generate libraries, which were single-end sequenced using the NextSeq500 High Output kit V2 (Illumina) for 75 cycles. Data are deposited in the NCBI-GEO database, accession number GSE193978. STAR (v2.5.1b) [1] was used to align the sequence data from FASTQ files, to the Human g1kv37 reference genome, and quality control performed with FastQC [2]. Downstream analysis was performed in R [3]. HTseq-count (v0.61) was used for transcript quantification [4]. Lowly expressed transcripts for which counts did not exceed 50 across all samples were removed. Data were normalised using R package DESeq2 (1.30.0) [5]. For the MaSigPro [6] analysis, due to the number of timepoints, a quadratic regression model (degrees of freedom = 2) was executed. False discovery correction was done using Benjamini-Hochberg method with an adjusted p-value of < 0.05 deemed significant. Molecular Degree of Perturbation (MDP) analysis was performed using the R package mdp [7]. The R package tmod [8] was used to perform modular analysis on the genes found to be differentially expressed between clinical groups using its HGtest function against all genes as a background. Modules with an adjusted p-value < 0.05 were deemed significant. Modular activity was calculated by summing the differential expression of genes in TB/DM relative to TB-only within a module and then dividing by the number of genes within that module. The g:profiler webtool [9] was used for gene ontology and pathway analyses gene lists.

Reverse-Transcriptase Multiplex Ligation-dependent Probe Amplification (dcRT-MLPA)

Reverse-Transcriptase Multiplex Ligation-dependent Probe Amplification (dcRT-MLPA) was performed using the SALSA MLPA kit (MRC-Holland) as described elsewhere [10]. RT primers and half-probes were designed by Leiden University Medical Centre (LUMC, Leiden, the Netherlands) and comprised sequences for 4 housekeeping genes and 144 selected key immune-related genes to profile specific compartments of the human immune response (Supplementary Table S1). Briefly, 125 ng RNA was reverse transcribed to cDNA by incubation at 37°C for 15 min using Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Promega, Leiden, The Netherlands), gene-specific RT-primers (Sigma-Aldrich, Zwijndrecht, The Netherlands), followed by inactivation of the enzyme by heating at 98°C for 2 min. The left- and right-hand half probes were hybridized to the cDNA at 60°C overnight, followed by ligation at 54°C for 15 minutes using ligase-65 (MRC-Holland), and inactivation by heating at 98°C for 5 min. Ligated probes were amplified by

PCR (33 cycles at 95°C for 30 seconds, 58°C for 30 seconds and 72°C for 60 seconds, followed by one cycle at 72°C for 20 minutes). PCR products were 1:10 diluted in Highly deionized (Hi-Di) formamide (ThermoFisher) containing 400HD Rhodamine X (ROX) fluorophore size standard (ThermoFisher). PCR products were denatured at 95°C for 5 min, stored immediately at 4°C and analyzed on an Applied Biosystems 3730 capillary sequencer in GeneScan mode (BaseClear, Leiden, The Netherlands). Trace data were analyzed using GeneMapper software 5 (Applied Biosystems, Warrington, UK). The areas of each assigned peak (arbitrary units) were exported for analysis in R (version 3.6.3). Data were normalized to housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and signals below the threshold value for noise cutoff in GeneMapper ($\log_2$ transformed peak area 7.64) were assigned the threshold value for noise cutoff.

dcRT-MLPA data were analysed to identify differentially expressed genes (DEGs) between groups at diagnosis by the non-parametric Mann-Whitney U-test with Benjamini-Hochberg correction for multiple testing. Ingenuity Pathway Analysis (IPA-60467501) (QIAGEN, Hilden, Germany) was used to explore interactive networks between the DEGs. Molecular Degree of Perturbation (MDP) analysis (mdp R package) [7], Partial Least Squares – Discriminant Analysis (PLS-DA) (mixOmics R package) [11] and Pearson correlations of gene expression ($\log_2$ FC) versus healthy control were performed in R version 4.0.2. Longitudinal changes in gene expression levels from diagnosis (baseline) to 6 months (Indonesian cohort) or 12 months (South African cohort) were assessed by means of linear mixed models for repeated measurements over time. Models were fitted to base 2 logarithm transformed measurements using lme4 package in R using the lmer function [12]. Group-time interactions were included as fixed effects and the patients ID-time interactions were included as random effects. For the South African cohort, we forced a b-spline at 6 months, which enabled us to identify altered gene expressions during treatment (0-6 months) as well as altered gene expression after treatment (6-12 months). Time was coded as 0 for the first timepoint (diagnosis) and as a continuous variable for the time difference between the two time points. P-values were adjusted for multiple testing using the False Discovery Rate (FDR) method of Benjamini-Hochberg [13]. An adjusted p-value < 0.05 and a $\log_2$ -fold change (FC) < -0.6 and > 0.6 were set as threshold for the identification of differentially expressed genes (DEGs). Genes that were below the detection limit in >90% of the samples per cohort were excluded from analysis. Signatures with the best discriminatory capability were identified using logistic regression with lasso regularization (glmnet R package) [14]. Leave-One-Out Cross Validation (LOOCV) and Train-Test Split (TTS) were used to assess the performance of the trained regression models. The classifying performance of the models were assessed by evaluating sensitivity, specificity, Receiver Operating Characteristic (ROC) curve, and Area Under the

ROC Curve (AUC) with 95% Confidence Interval (CI), and box-and-whiskers-plots representing the predicted probability for each class were used to evaluate the classifying performance of the models.

Supplementary References

1. Dobin A, Davis CA, Schlesinger F, et al. STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* 2013; 29(1): 15-21.
2. Andrews S. FastQC: a quality control tool for high throughput sequence data. Available at: <http://www.bioinformatics.babraham.ac.uk/projects/fastqc>.
3. R Core Team. R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing, 2013.
4. Anders S, Pyl PT, Huber W. HTSeq--a Python framework to work with high-throughput sequencing data. *Bioinformatics* 2015; 31(2): 166-9.
5. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* 2014; 15(12): 550.
6. Nueda MJ, Tarazona S, Conesa A. Next maSigPro: updating maSigPro bioconductor package for RNA-seq time series. *Bioinformatics* 2014; 30(18): 2598-602.
7. Lever M RP, Nakaya H. mdp: Molecular Degree of Perturbation calculates scores for transcriptome data samples based on their perturbation from controls. R package version 1.12.0. Available at: <https://mdp.sysbio.tools/>.
8. Weiner 3rd J, Domaszewska T. tmod: an R package for general and multivariate enrichment analysis. *PeerJ Preprints* 2016; 4: e2420v1
9. Raudvere U, Kolberg L, Kuzmin I, et al. g:Profiler: a web server for functional enrichment analysis and conversions of gene lists (2019 update). *Nucleic Acids Res* 2019; 47(W1): W191-W8.
10. Joosten SA, Goeman JJ, Sutherland JS, et al. Identification of biomarkers for tuberculosis disease using a novel dual-color RT-MLPA assay. *Genes Immun* 2012; 13(1): 71-82.
11. Kim-Anh Le Cao FR, Ignacio Gonzalez, Sebastien Dejean. mixOmics: Omics Data Integration Project. R package version 6.1.1. Available at: <https://CRAN.R-project.org/package=mixOmics>.
12. Bates D, Mächler M, Bolker BM, Walker SC. Fitting linear mixed-effects models using lme4. *Journal of Statistical Software* 2015; 67.
13. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal statistical society: series B (Methodological)* 1995; 57(1): 289-300.
14. Friedman J HT, Tibshirani R. Regularization Paths for Generalized Linear Models via Coordinate Descent. *Journal of Statistical Software* 2010; 33(1), 1-22.

Chapter 3

Transcriptional profiles predict treatment outcome in patients with tuberculosis and diabetes at diagnosis and at two weeks after initiation of anti-tuberculosis treatment

Cassandra L.R. van Doorn*, Clare Eckold*, Katharina Ronacher, Rovina Ruslami, Suzanne van Veen, Ji-Sook Lee, Vinod Kumar, Sarah Kerry-Barnard, Stephanus T. Malherbe, Léanie Kleynhans, Kim Stanley, Philip C. Hill, Simone A. Joosten, Reinout van Crevel, Cisca Wijmenga, Julia A. Critchley, Gerhard Walzl, Bakti Alisjahbana, Mariëlle C. Haks, Hazel M. Dockrell, Tom H.M. Ottenhoff, Eleonora Vianello[#] and Jacqueline M. Cliff[#], on behalf of the TANDEM Consortium

**These authors contributed equally*

[#]These authors contributed equally

Abstract

Background

Globally, the tuberculosis (TB) treatment success rate is approximately 85%, with treatment failure, relapse and death occurring in a significant proportion of pulmonary TB patients. Treatment success is lower among people with diabetes mellitus (DM). Predicting treatment outcome early after diagnosis, especially in TB-DM patients, would allow early treatment adaptation for individuals and may improve global TB control.

Methods

Samples were collected in a longitudinal cohort study of adult TB patients from South Africa (n= 94) and Indonesia (n=81), who had concomitant DM (n=59), intermediate hyperglycaemia (n=79) or normal glycaemia/no DM (n=37). Treatment outcome was monitored, and patients were categorized as having a good (cured) or poor (failed, recurrence, died) outcome during treatment and 12 months follow-up. Whole blood transcriptional profiles before, during and at the end of TB treatment were characterized using unbiased RNA-Seq and targeted gene dcRT-MLPA.

Findings

We report differences in whole blood transcriptome profiles, which were observed before initiation of treatment and throughout treatment, between patients with a good versus poor TB treatment outcome. An eight-gene and a 22-gene blood transcriptional signature distinguished patients with a good TB treatment outcome from patients with a poor TB treatment outcome at diagnosis (AUC=0.815) or two weeks (AUC=0.834) after initiation of TB treatment, respectively. High accuracy was obtained by cross-validating this signature in an external cohort (AUC=0.749).

Interpretation

These findings suggest that transcriptional profiles can be used as a prognostic biomarker for treatment failure and success, even in patients with concomitant DM.

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Introduction

With more than 10 million new cases and approximately 1.5 million deaths annually, tuberculosis (TB), which is caused by *Mycobacterium tuberculosis* (*Mtb*), continues to be a major global health threat (1). Upon infection with *Mtb*, 5-10% of adults develop active disease during their lifetime and one quarter of the world's population is estimated to be latently infected with *Mtb* (LTBI) (1). The global TB treatment success rate is only about 85% and even lower in patients with multi-drug resistant TB or with comorbidities like HIV or diabetes mellitus (DM) (1-3), resulting in a significant number of patients with poor clinical outcomes.

DM triples the risk of developing active TB (4) and increases the risk of poor clearance of the infection following TB treatment (5-7). In 2020, 0.37 million TB cases were estimated to suffer from DM comorbidity (1). Around 85-95% of all DM cases are attributed to type-2 diabetes mellitus (T2DM) (8). Since global DM prevalence is estimated to rise from 463 million people in 2019 to 700 million in 2045 (9), in particular in areas where TB is endemic, there is increasing concern about the consequences of the rising DM prevalence for global TB control (1). The mechanisms underlying DM-induced TB treatment failure remain, however, poorly understood.

Prediction of TB treatment failure based on sputum-smear microscopy and mycobacterial culture lacks sensitivity (10) and depends on the quality of sputum samples, which are difficult to collect and are frequently inconsistent in quality (11-13). As well as more advanced sputum-based diagnostics, monitoring of whole blood transcriptomics may be an additional, complementary but independent, method to monitor treatment responses, possibly with increased sensitivity (14). Numerous studies have reported transcriptional biomarker profiles for active TB and response to TB treatment using whole-blood or PBMCs in settings with varying TB incidence (15-20). In addition, multiple studies have demonstrated the predictive potential of host gene biomarkers in identifying patients at risk of developing active TB, relapse and treatment failure (21-28). Together, these studies showed that gene signatures may have utility at predicting TB treatment success versus failure early after TB diagnosis, providing a significant improvement over the currently used, low sensitivity, conversion to negative sputum-based culture testing (10). Despite the high incidence of DM and pre-DM among TB patients in TB-endemic settings (7, 29-31), only a few studies have identified or validated such signatures in TB patients with DM or hyperglycemia (32, 33).

Characterizing transcriptomic profiles may improve our understanding about immunological pathways that are involved in DM-associated TB pathology, and monitoring treatment success and failure in TB patients with concomitant DM is key to combatting the tuberculosis-diabetes (TB-DM) co-epidemic. Although the blood transcriptome profile

of TB-DM patients is more similar to TB patients than to DM patients, suggesting a dominant influence of active TB infection, we and others recently demonstrated significant differences in the blood transcriptome of TB-DM patients compared to TB patients (32, 33). Additionally, the transcriptomic profiles of patients with TB-related intermediate hyperglycemia (TBrel-IH) are similar to the profiles of TB-DM patients (32). Importantly, we also showed that DM comorbidity lowered the performance of published diagnostic biomarker signatures (32). Therefore, there is a need for biomarkers that predict treatment outcome in heterogeneous TB populations, including TB-DM patients.

The aim of the current study was to identify a blood transcriptional gene signature to predict TB treatment outcome at an early stage after initiation in a TB population including patients with varying glycaemia and DM status. We combined an unbiased RNA-Seq approach and a selective dcRT-MLPA approach (a multiplex RT-PCR platform) as two independent strategies to identify gene signatures with high discriminatory power to distinguish patients with a good TB treatment outcome from patients with a poor TB treatment outcome. Host gene biomarker profiles to identify TB treatment success or failure could facilitate the evaluation of new TB drugs and improve clinical surveillance of TB patients, even in settings with high DM incidence.

Methods

Study participant recruitment, classification and treatment

Adult pulmonary TB patients were recruited between January 2014 and February 2017, as part of the TANDEM project (29) in two locations: Bandung in Indonesia (UNPAD) and Cape Town in South Africa (SUN) (Supplementary Figure S1). All TB patients were newly diagnosed and microbiologically confirmed, and included people with TB-DM. The TB-DM group included participants with both pre-diagnosed DM and newly identified DM, with new diagnosis based on a laboratory HbA1c test $\geq 6.5\%$ with a confirmatory HbA1c test $\geq 6.5\%$ or fasting blood glucose ≥ 7 mmol/L at TB diagnosis (29), followed by a further HbA1c test $\geq 6.5\%$ after 6 months of TB treatment. The TB patients without DM included people with a normal glycaemic index (laboratory HbA1c $< 5.7\%$) at TB diagnosis ("TB-only"). Patients whose HbA1c test results were $\geq 5.7\%$ and $< 6.5\%$ were deemed to have intermediate hyperglycaemia. In South Africa, healthy community controls (HC) without TB or DM were also recruited at baseline: HC were all sputum smear and culture negative, had normal chest x-rays and had laboratory HbA1c $< 5.7\%$. The age range and sex balance were similar across the HC and the TB patients, analysed either as all TB patients combined, or for separate treatment outcome groups (Table 1). Multi-drug-resistant TB, HIV positivity, pregnancy, serious co-morbidity and corticosteroid use were exclusion criteria.

TB patients received standard first line TB treatment according to WHO Guidelines. Microbiological measures recorded at baseline and throughout treatment included sputum smear and culture, with time to positivity (TTP) in mycobacteria growth indicator tubes (MGIT) also assayed in South Africa. TB patients were classified based on their TB treatment outcome: “poor TB treatment outcome” included those patients who died, failed initial treatment (remained sputum positive at six months) or experienced TB-recurrence in the 12 month clinical follow-up period post treatment, whilst those with “good TB treatment outcome” had successful TB treatment without subsequent recurrence. Patients for whom the outcome data were missing were not included in downstream analyses. Most TB-DM patients received local standard of care DM treatment following national guidelines outside of the TANDEM study, which largely involved metformin and glibenclamide prescription, whilst a subgroup in Indonesia received more intensive education and counselling, glucose and HbA1c monitoring and treatment adjustment through TB treatment as part of a pragmatic randomised control trial (34), in which they were assessed at weeks 1, 2 and 4 and then monthly throughout TB treatment, and treatment optimised at each visit, leading to better HbA1c control at 6 months in this group.

External validation data

Data from a prospective cohort study of adult pulmonary TB cases were used for external validation (28). The cohort consists of pulmonary TB patients that were recruited in Palamaner and Kuppam Taluks, Chittoor district, Andhra Pradesh, India between September 2010 and April 2012. Pulmonary TB was radiologically confirmed. Patients received standard TB treatment and were followed for six months. For this study, data from 67 participants were available. This cohort was constituted of 55 (82%) males and 12 (18%) females with a mean age of 43 (18-75) years. Among the 67 patients, 45 (67%) patients had successful TB treatment (“good TB treatment outcome”), while 22 (33%) failed treatment (“poor TB treatment outcome”). Diabetes was recorded in 9 (13%) participants, all of whom had successful TB treatment.

Ethics Statement

The study was approved by the London School of Hygiene & Tropical Medicine Observational Research Ethics Committee (6449), the SUN Health Research Ethics Committee (N13/05/064) and the UNPAD Health Research Ethics Committee, Faculty of Medicine, Universitas Padjadjaran (number 377/UN6.C2.1.2/ KEPK/ PN), and participants gave written informed consent.

RNA sample collection and extraction

Patient samples were collected prior to initiation of treatment (diagnosis), at weeks 2, 4, 8, 16 and 24 through treatment, and at 12 months after TB diagnosis (6 months after treatment completion), and from HC at baseline only. Venous blood (2.5ml) was collected into PAXgene Blood RNA Tubes (PreAnalytiX). Total RNA was extracted using RNeasy spin columns (Qiagen) and quantified by Nanodrop (Agilent). The LabChip GX HiSens RNA system (PerkinElmer) was used for quality assessment of samples processed by RNA-Seq.

Unbiased RNA-Seq of global gene expression

Samples collected at TB diagnosis and weeks 2, months 2, and months 6 from the first 63 participants recruited were analysed by RNA-Seq (Table 1). Libraries were generated using the poly-A tail Bioscientific NEXTflex-Rapid-Directional mRNA-Seq method with the Caliper SciClone. Single-end sequencing was performed using the NextSeq500 High Output kit V2 (Illumina) for 75 cycles. Sequence data from FASTQ files were aligned to the Human g1kv37 reference genome, using STAR (v2.5.1b) (35). Quality control was performed with FastQC (36), while transcript quantification was performed using HT-seq count (v0.61) (37): lowly expressed transcripts (<50 counts across all samples), were removed from the downstream analysis. RNA-Seq data were normalised using DESeq2 (v1.30.0) (38).

Dual-color Reverse-Transcriptase Multiplex Ligation-dependent Probe Amplification (dcRT-MLPA)

Dual-color reverse-Transcriptase Multiplex Ligation-dependent Probe Amplification (dcRT-MLPA) was performed on all samples to identify blood transcriptional profiles as described previously (39). Brief descriptions are provided in the Supplementary Information. RT primers and half-probes were designed by Leiden University Medical Centre (LUMC, Leiden, The Netherlands) and encompassed sequences for 144 selected key immune-related genes to profile the innate, adaptive and inflammatory immune responses (Supplementary Table S1), and four housekeeping genes (*GAPDH*, *ABR*, *GUSB*, *B2M*). Genes with an adjusted p-value <0.05 (Benjamini-Hochberg (40)) and a log₂-fold change (FC) <-0.6 and >0.6 were considered differentially expressed genes (DEGs). Genes that were below the detection limit in >90% of the samples per cohort were excluded from analysis.

Data analysis and Statistics

Statistical analyses to compare participant demographics were carried out using GraphPad Prism 8 software (Graphpad Software, San Diego, CA, USA). Data for most variables were not normally distributed, as determined by the D'Agostino & Pearson and the Shapiro-Wilk tests, thus non-parametric statistical comparison methods were employed for analysis of the study participants. For continuous measures, a Mann-Whitney U-test was

used when comparing two groups and a Kruskal-Wallis test when comparing three groups. For non-continuous measures, the Chi-square test was used. P-values <0.05 were considered significant.

Molecular Degree of Perturbation (MDP) analysis was performed to quantify the molecular distance of samples within a group compared to a reference group (“healthy controls” or “diagnosis”). MDP scores were calculated by R using the *mdp* R package (41), and differences between the mean ranks of the groups were assessed by Mann-Whitney U test followed by Benjamini-Hochberg False discovery correction (40). Cell population estimates were calculated using the Cell-type Computational Differential Estimation cellCODE (42) R package which enables the prediction of cellular composition without external measurement, and which would allow prediction of cell-specific gene expression if samples were heterogeneous: the Immune Response In Silico:IRIS (43) and Differentiation Map:DMAP (44) data sets were used as references in CellCODE analysis. Modular analysis was performed using the R package tmod (45) and its HGtest method, with DEGs used as the foreground and all genes used as the background signal. Modular analysis aims to reduce the complexity of transcriptomic datasets, grouping together sets of genes which are co-expressed and behave in a similar manner across experimental designs.

Principal Component Analysis (PCA) and Pearson correlation (R package stats, function *prcomp* and *cor.test*, respectively) were used to evaluate the influence of sex, age, BMI, and HbA1c levels on the gene expression.

Differential expression analysis (DEA) was performed in the RNA-Seq dataset in R using the MaSigPro package (46) to characterise longitudinal differential gene expression of genes measured by RNA-Seq: this followed a two-step regression method, firstly using the least squared technique and also performing a false discovery rate correction, and secondly a stepwise regression to find genes with significant temporal expression changes, significant differences between clinical groups and to find clusters of genes with similar expression behaviour. A quadratic regression model was executed due to the number of timepoints analysed.

Longitudinal DEA of genes measured by dcRT-MLPA was assessed by means of linear mixed models for repeated measures over time using lme4 package in R (47). A Benjamini-Hochberg False discovery correction was performed, with an adjusted p-value of <0.05 deemed significant. Non-parametric Mann-Whitney U-test followed by Benjamini-Hochberg correction was performed to identify DEGs between patients who had a good and poor TB treatment outcome. Correlations were evaluated using Pearson’s correlation coefficient.

TB treatment outcome signatures based on dcRT-MLPA data were identified in TB

patients from South Africa and Indonesia using Recursive Feature Elimination (RFE) (48) and Random Forest (RF). Because the number of patients with a good TB treatment outcome was considerably larger than those with a poor TB treatment outcome (poor, $n=38$; good, $n=134$), a random down-sampling technique as well as a Synthetic Minority Oversampling Technique (SMOTE) were applied to balance the classes (i.e. “good TB treatment outcome” and “poor TB treatment outcome”) of the dataset (49). RF was performed as machine learning algorithm on the dataset including the selected genes and the performance of gene signatures was evaluated by Leave-One-Out Cross Validation (LOOCV) (50, 51). We assessed the classifying performance of the model by evaluating Receiver Operating Characteristic (ROC) curve and Area Under the ROC Curve (AUC) with 95% Confidence Interval (CI). DeLong test was used to compare correlated ROC curves. An extended description of the data-analysis methods is provided in the Supplementary Information.

Role of Funders

Funders had no role in study design, data collection, data analyses, data interpretation, writing of the report and decision to submit the paper for publication.

Results

Study Design and Cohort

Pulmonary TB patients were recruited into the prospective longitudinal study in South Africa ($N=94$) and Indonesia ($n=81$), and followed up through standard treatment and for the following 12 months. Altogether, 38 TB patients of the 175 recruited had a “poor TB treatment outcome”, with 6 patients dying, 26 failing treatment (based on continued sputum smear or culture positivity at month 6), and 6 experiencing recurrences in the subsequent 12 months (Supplementary Figure S1). The “poor TB treatment outcome” rates were similar in the two sites (Table 1). The median age of the patients was equal in patients with either a good or poor TB treatment outcome (median = 47 years), with a higher proportion of males with a poor TB treatment outcome (67%) than a good TB treatment outcome (56%). While there was a slightly higher proportion of patients with DM in the poor TB treatment outcome group (15/39; 38%) than the good TB treatment outcome group (44/137; 32%), the difference in DM status across the outcome groups was not statistically significant in Chi squared test ($P=0.55$; Table 1). By definition, there were significant differences in DM status and HbA1c between TB patients and HC, as the latter were recruited based on their normal glycaemic status. There was no evidence that those who had a poor TB treatment outcome had more severe TB at diagnosis, with similar sputum bacterial loads (as measured by TTP) in TB patients from South Africa and similar sputum smear grade in Indonesia across the good and poor TB treatment outcome groups.

The BMIs in the TB patients with good or poor treatment outcome were not significantly different to each other ($P=0.835$), but were highly significantly lower ($P<0.0001$) than the HC group when tested as separate groups or as a combined TB patient group, which is expected as TB patients are often underweight.

TABLE 1 | Study Participant Demographics.

Characteristic	Country	TB Patients			Healthy Controls	P-value Good vs Poor outcome 2-way comparison	P-value Combined TB groups vs HC 2-way comparison
		Good Outcome	Poor Outcome	Combined TB groups			
Total Number of Participants	S Africa	76	18	94	27	-	-
	Indonesia	61	20	81	0	-	-
	All	137	38	175	27	-	-
Age in years, median (range)	S Africa	46 (22-68)	42 (19-55)	45 (19-68)	42 (30-70)	0.258	0.728
	Indonesia	49 (25-73)	49 (35-68)	49 (25-73)	-	0.96	-
	All	47 (22-73)	47 (19-68)	47 (19-73)	42 (30-70)	0.619	0.1286
Sex, % male (No. male/female)	S Africa	58 (44/32)	67 (12/6)	60 (56/38)	52 (14/13)	0.495	0.474
	Indonesia	53 (32/29)	65 (13/7)	56 (45/36)	-	0.258	-
	All	56 (76/61)	66 (25/13)	58(101/74)	52 (14/13)	0.212	0.567
Number with Diabetes / Intermediate Hyperglycaemia / Normal glycaemia (%)	S Africa	13/49/14	4/11/3	17/60/17	0/0/27	0.8785	<0.0001
	Indonesia	31/16/14	11/3/6	42/19/20	-	0.559	-
	All	44/65/28	15/14/9	59/79/37	0/0/27	0.541	<0.0001
HbA1c median (range)	S Africa	6.0 (4.9-14.3)	6.0 (4.8-14.1)	6.0 (4.8 – 14.3)	5.3 (4.8-6.4)	0.614	<0.0001
	Indonesia	8.15 (4.9-17.1)	7.1 (5.1-14.1)	7.8 (4.9 – 17.1)	-	0.561	-
	All	6.0 (4.9-17.1)	6.1 (4.8-14.1)	6.0 (4.8 – 17.1)	5.3 (4.8-6.4)	0.989	<0.0001
BMI at TB diagnosis; median (range)	S Africa	18.7 (13.9-32.3)	18.3 (13.7-31.2)	18.7 (13.9-32.3)	23.2 (17.4-45.2)	0.903	<0.0001
	Indonesia	19.7 (13.8-33.3)	18.8 (16.3-27.3)	19.6 (13.8-33.3)	-	0.843	-
	All	19.1 (13.8-33.3)	18.8 (13.7-31.2)	19.0 (13.7-33.3)	23.7 (17.4-45.2)	0.835	<0.0001
TTP(days) at TB diagnosis: Median (range) (missing values)	S Africa	6 (1-21) (18)	6 (3-21) (3)	6 (1-21) (21)	N/A	0.820	-
Smear Grade at diagnosis number: 3+/2+/1+/-scanty/negative	Indonesia	16/23/14/2/6	8/4/5/2/1	24/27/19/4/7	N/A	0.364	-
Sputum conversion at Month 2: number yes/no (missing values)	S Africa	49/19 (8)	10/6 (2)	59/25 (10)	N/A	0.452	-
	Indonesia	44/15 (2)	10/8 (2)	54/23 (4)	N/A	0.123	-
	All	93/34 (10)	20/14 (4)	113/48 (14)	N/A	0.103	-
Outcome classification: Cured /Recurrence/Failed/Died	S Africa	76/0/0/0	0/4/10/4	76/4/10/4	N/A	<0.0001	-
	Indonesia	61/0/0/0	0/2/16/2	61/2/16/2	N/A	<0.0001	-
	All	137/0/0/0	0/6/26/6	137/6/26/6	N/A	<0.0001	-
RNASeq subset	S Africa	26	6	32	0	-	-
	Indonesia	23	8	31	0	-	-
	All	49	14	63	0	-	-
MLPA subset	S Africa	76	18	94	27	-	-
	Indonesia	58	19	77	0	-	-
	All	135	37	172	27	-	-

Combined TB Groups is the combination of TB patients with a Good or Poor Outcome. Continuous variables were compared by Mann-Whitney U test (2 groups) or Kruskal-Wallis test (3 groups); non-continuous variables by Chi-square test.

The transcriptomic response in patients with a poor and good TB treatment outcome

The holistic unbiased analysis of gene expression in TB patients with good or poor TB treatment outcomes by RNA-Seq approach was performed on a subset of study participants, who were the first 63 recruited participants (Table 1). Molecular Degree of Perturbation (MDP) analysis revealed that there were significant changes in global gene expression in patients with a good TB treatment outcome continuously through TB treatment, reflecting treatment response (Figure 1A). Gene expression perturbation was also evident in patients who had a poor TB treatment outcome, although the sample score was higher at diagnosis compared to patients who had a good TB treatment outcome. This represents differences at the transcriptomic level between patients with a good versus a poor TB treatment outcome, already evident before initiation of TB treatment. This was followed by less change over time in response to TB treatment in the poor TB outcome group.

Next, we focused our molecular distance analysis on 144 TB-associated genes as measured by dcRT-MLPA, which was performed on all study participants (n=199) (Table 1). There were significant changes in global gene expression already observable two weeks after initiation of TB treatment in patients with a good TB treatment outcome, but not in patients with a poor TB treatment outcome (Figure 1B), reflecting a delayed TB treatment response in the latter group. Gene expression perturbation normalized towards levels of healthy controls throughout treatment in patients with a good TB treatment outcome, but not in patients with a poor TB treatment outcome (Supplementary Figure S2). However, despite the substantial treatment response in patients with a good TB outcome, gene expression perturbation did not completely normalize to levels of healthy controls.

Together, these data suggest that there was a different biosignature in those with good versus poor TB treatment outcomes, which was reflected by transcriptomic differences before initiation of TB treatment and by a delayed response to TB treatment in patients with a poor TB treatment outcome compared to patients with a good TB treatment outcome.

Global Differential Expression Analysis in patients with good or poor TB treatment outcome

The changes in gene expression in the RNA-Seq dataset through time and between the patients with a good or poor TB treatment outcome were analyzed by MaSigPro, an R package designed for longitudinal RNA-Seq data, initially in the pooled South African and Indonesian cohorts. The MaSigPro regression modelling tool treats time as a quantitative variable, so as well as differentially expressed genes (DEGs) being detected, the changes in trends and magnitude are also included. Thus, using MaSigPro, we could determine the change in gene expression over time and also between different treatment groups (Figure

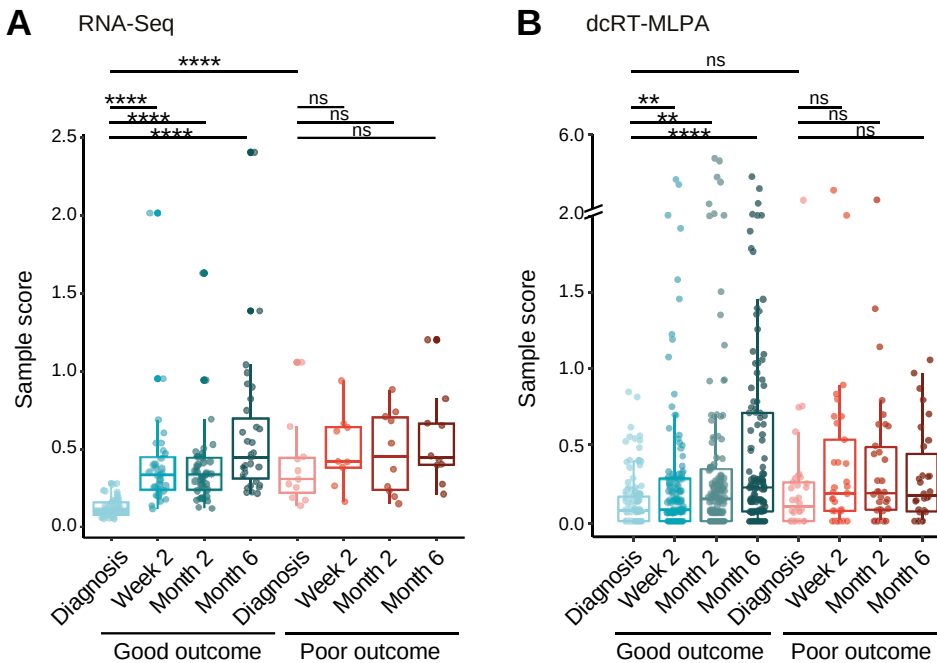


FIGURE 1 | MDP plots representing the change in gene expression perturbation in TB patients categorized based on TB treatment outcome. Full blood transcriptomes from TB patients who had a good or poor TB treatment outcome were determined by (A) RNA-Seq and by (B) dcRT-MLPA. The extent of overall difference in gene expression, relative to the median of expression at diagnosis in those who had a good TB treatment outcome, was calculated for individual patients at the timepoints shown. The bars and whiskers show the median and data within the Q1-1.5 x inter quartile range (IQR) and Q3+1.5 x IQR interval. Differences were significant by Mann-Whitney U-test with Benjamini-Hochberg correction for multiple testing. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

2, Supplementary Table S4). The genes differentially expressed through treatment in the pooled analysis separated into nine clusters, with variable patterns of expression over time and between TB patients with good or poor TB treatment outcome. Some clusters (2,5,6) contained genes which were different between the groups at all time points, whereas other clusters (1,3,4,5,7,8,9) were similar at some timepoints and more divergent at others (Figure 2, Table 2). Similarly, cluster analysis of gene expression in the cohorts *separately* revealed nine gene clusters, with gene clusters increasing (South Africa: 1,2,3,5, Indonesia: 2,5,7) or decreasing (South Africa: 4,6,7,8, Indonesia: 1,3,4,6,8,9) through time (Supplementary Figure S3, Supplementary Table S2 and S3), and with higher expression in TB patients with a poor outcome in gene clusters (South Africa: 1,3,4,5,7,8,9, Indonesia: 2,6,8,9) and with a good outcome in gene clusters (South Africa: 2,6, Indonesia: 1,3,4). Importantly, these differences in gene expression through time were observed in all TB patient groups, irrespective of their DM status.

The number of transcripts within each cluster in the combined pooled cohort analysis ranged from 4 to 47 (Table 2), with the majority of genes identified in all clusters encoding proteins. There were also various regulatory transcripts in some clusters, including long non-coding RNAs, miRNA, snoRNA, retained introns, as well as antisense, nonsense-mediated decay, overlapping senses and sense intronic transcripts. To understand the biological function of the differentially expressed genes (DEGs), the transcripts within each cluster were analysed using the g:COST tool within the g:Profiler application (52), to determine significant enrichment of genes in Gene Ontology (GO) molecular function, cellular component and biological process categories, as well as in curated biological pathways from KEGG and Reactome databases and the CORUM protein database. Genes in cluster 2 were largely involved in B cell receptor signalling, seen in the GO and pathway analyses, and these were more highly expressed in people who had a poor TB treatment outcome, with increasing expression through treatment. This upregulation of genes involved in B cell function, particularly those involved in earlier stages of B cell development, was not related to the overall number of B cells in the samples, as predicted from the samples using CellCODE analysis package which showed that there were no

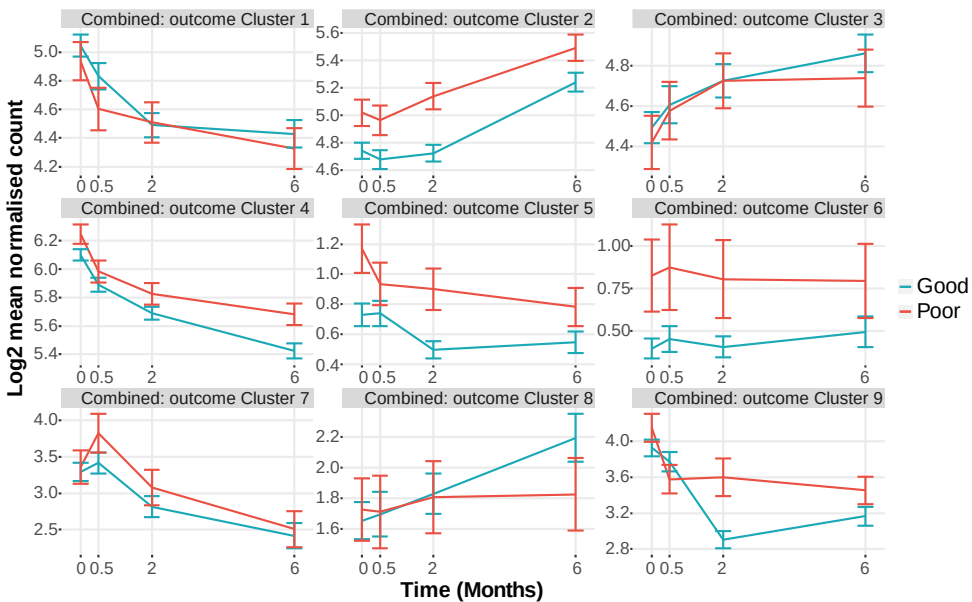


FIGURE 2 | MaSigPro analysis of TB patients with good or poor TB treatment outcome, across combined South African and Indonesian cohorts. Plots show hierarchical clusters of genes in patients with a good (blue) or poor (red) TB treatment outcome. Bars show mean $\pm$ 1 SEM. Data were filtered to remove lowly abundant transcripts prior to analysis.

TABLE 2 | Clusters of genes differentially expressed between TB patients with good or poor TB treatment outcomes to TB treatment in MaSigPro analysis of combined RNA-Seq data from South Africa and Indonesia.

Cluster Number	Overall pattern	Number of gene transcripts	Gene Function				Top Functions from g:Profiler** with adjusted P<0.05
			Protein Coding	Processed Transcript	Pseudo-gene	Regulatory RNAs*	
1	↓ through treatment; Higher in Good at M0.5	26	20	1	0	5	GO:MF – Opsonin Binding; GO:CC – Intracellular Vesicles; Endomembrane System
2	↑ through treatment; Higher in Poor throughout	14	13	0	0	1	GO:BP – B cell receptor (BCR) signaling; GO:CC – BCR complex KEGG – BCR signaling; primary immunodeficiency; REAC – BCR signaling WP – BCR signaling CORUM – CIN85-BLNK complex
3	↑ through treatment; Higher in Good at M6	25	17	2	0	6	<i>No significant results</i>
4	↓ through treatment; Higher in Poor throughout	47	37	2	2	6	GO:CC – Arp2/3 complex; KEGG – Shigellosis; E.coli, Yersinia, Salmonella infection; Endocytosis; REAC – Ephrin signaling; Rho GTPases activate WASPs and WAVES; TF – ZNF544 CORUM – Arp2/3 complex
5	Small ↓ through treatment; Higher in Poor throughout	11	6	1	1	3	GO:MF – L-tyrosine transmembrane transporter activity; GO:BP – positive regulation of fatty acid transport.
6	No change; Higher in Poor throughout	4	0	0	3	1	<i>No significant results</i>
7	↑ to M0.5, then ↓; Higher in Poor at M0.5	4	4	0	0	0	GO:BP – mitotic cell cycle process WP – Retinoblastoma Gene in Cancer
8	↑ in Good through treatment; No change in Poor	7	3	0	2	2	GO:MF - RNA polymerase III activity
9	↓ through treatment; Much greater change in Good	10	10	0	0	0	GO:MF – immunoglobulin receptor binding GO:BP – phagocytosis, recognition; complement activation, classical pathway; Immunoglobulin mediated immune response; B cell activation GO:CC – immunoglobulin complex; E/C space; plasma membrane REAC – Classical antibody-mediated complement activation; FCGR activation; phagocytosis.

*Retained introns, Antisense, LncRNA, miRNA, nonsense-mediated decay, sense overlapping, sense intronic, snoRNA,

**Redundant G:Profiler results are not shown

significant differences in the proportions of any of the predicted cell types (corrected $P > 0.05$; Supplementary Figure S4). Cluster 9 was predominantly composed of immunoglobulin transcripts, whose expression decreased much more substantially in patients with a good TB treatment outcome. The largest gene cluster (4) was enriched with genes involved in actin remodelling, including the Arp 2/3 complex, and in pathways related to infections with bacteria such as *Shigella*, *E. coli*, *Yersinia* and *Salmonella*. Cluster 7 contained genes related to mitotic cell division, and these were more highly expressed in patients with a poor TB treatment outcome (Table 2). These analyses were also performed using the DAVID online tool (53), and similar results were obtained (not shown). The DEGs found in the combined and separate cohort MaSigPro analyses were used as a foreground against all genes in a modular analysis using the Tmod package, which gives biological function to a gene list. It showed an upregulation of genes involved in B cell function in good versus poor TB treatment outcomes, in both the Indonesian and South African cohorts (Supplementary Table S5).

Differential Expression Analysis of focused gene expression in patients with good or poor TB treatment outcome

Next, we focused our DEA on 144 genes that previously have been associated with TB (39) using dcRT-MLPA (Supplementary Table S6). We decided to analyze longitudinal expression of genes (Figure 3) because no significant DEGs were detected by directly comparing patients with a good versus a poor TB treatment outcome at the indicated timepoints (Supplementary Figure S5). Kinetic profiling of DEGs identified 16 DEGs in patients with a good TB treatment outcome and 12 DEGs in patients with a poor TB treatment outcome. Genes associated with active TB (15, 20, 54) or risk of developing TB (22) were substantially downregulated (*GBP1*, *GBP2*, *GBP5*, and *IFITM3*) or upregulated (*GZLY* and *PRF1*) over time in TB patients regardless of their TB treatment outcome, reflecting transcriptomic response to TB treatment (Figure 3A and Supplementary Figure S6). Other genes associated with active TB were significantly down- or upregulated (*STAT2*, *MMP9*, *IRF7*, *IFI6*, *IFIT2*, *IFIT3*, and *CCR7*) during TB treatment in patients who had a good TB treatment outcome, but not in patients who had a poor TB treatment outcome, while genes such as *CD3E*, *PTPRCv1*, *NLRP1*, *BCL2* were upregulated in patients with poor TB outcome but not in patients with good TB outcome, confirming that altered changes in patients with a poor outcome could be observed using this methodology, despite the smaller sample size (15, 39, 54, 55). The expression of *TAGAP*, previously associated with active TB (55), was significantly increased during TB treatment in patients who had a poor TB treatment outcome. A high correlation between DEGs of patients who had a poor TB treatment outcome and DEGs of patients who had a good TB treatment outcome could be detected ($R=0.87$, $p<0.0001$), highlighting the challenge of discriminating patients with a

good versus a poor TB treatment outcome based on single genes (Supplementary Figure S7). Modular analysis showed that the gene profile of regulated genes was dominated by genes in the interferon (IFN) signaling pathway, especially in patients who had a good TB treatment outcome (Figure 3B). The longitudinal expression of DEGs identified by dcRT-MLPA showed a significant correlation with genes measured by RNA-Seq, highlighting the validity and reproducibility of our approach (Supplementary Figure S8).

Identification of a signature predicting TB treatment outcome

Machine learning algorithms were implemented on data obtained at each time point to develop biomarker panels to predict TB treatment outcomes at different stages of TB treatment. First, we aimed to identify gene signatures from RNA-Seq analysis on a subset of subjects, but we found a low performance of gene signatures generated on diagnosis, week two and month six (AUC=0.625, AUC=0.667 and AUC=0.615, respectively) to predict TB treatment outcome, potentially due to a low number of patients in the training and test set (Supplementary Figure S9A). The best performing model was built on month two resulting in an AUC of 0.8667 (Supplementary Figure S9A, Supplementary Table S7). We also tested an active TB disease biomarker signature, namely the three-gene Sweeney signature (20), to determine whether it resolved significantly more in those with a good TB treatment outcome than in those with a poor TB treatment outcome. This signature has previously been shown to persist in patients with persistent lung inflammation (26). However, in our RNA-Seq data, this signature revealed an AUC of 0.5333 (Supplementary Figure S9B) highlighting that the process behind poor TB treatment outcome cannot be predicted by expression of these three genes.

Next, we aimed to identify early correlates of TB treatment outcome by implementing machine learning algorithms on gene expression as measured by dcRT-MLPA. We focused our analysis on the identification of gene predictors at diagnosis and at week two that could possibly be used in future studies to predict the occurrence of poor or good TB treatment outcome before or early after TB treatment initiation, first by down-sampling the good TB treatment outcome class. The top eight ranked genes (*GBP1*, *FCGR1A*, *STAT1*, *IFITM3*, *BCL2*, *CCL4*, *TLR9*, *CD274*) from the diagnosis signature were used for RF machine learning model implementation (Table 3). Excitingly, the signature had a high predictive power to classify good and poor TB treatment outcome both in all TB patients irrespective of their DM condition and also only in the TB with concomitant diabetes group separately (TB-DM) (AUC=0.815 and AUC=0.792, respectively), and this was evident already before TB treatment initiation (Figure 4A and Supplementary Figure 10). Furthermore, the gene signature showed high performance in all TB patients from the separate cohorts (South Africa, AUC=0.845; Indonesia, AUC=0.744). By using this signature, we performed PCA and Pearson correlation analysis to verify that parameters

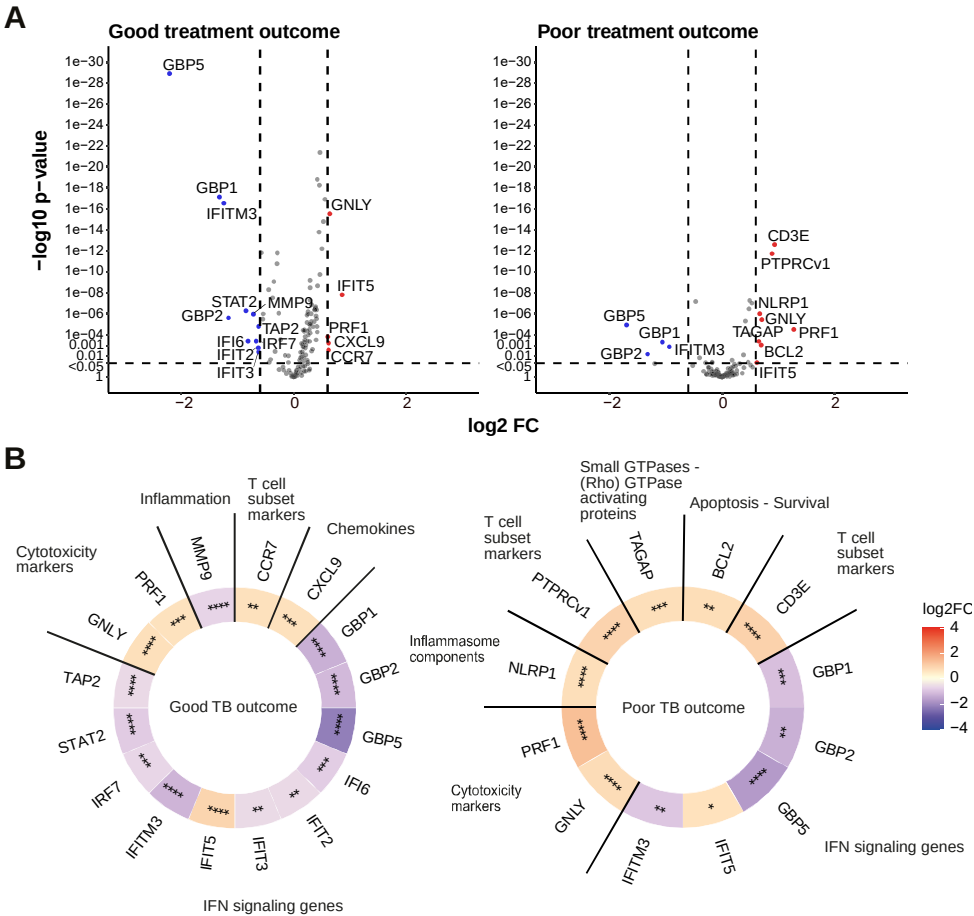


FIGURE 3 | DEA of all TB patients from the pooled (South African and Indonesian) cohorts categorized based on TB treatment outcome compared to their gene expression levels at diagnosis. (A) Volcano plots representing DEGs regulated during TB treatment of TB patients who had a good TB treatment outcome (left panel) or a poor TB treatment outcome (right panel). The y-axis scales of the plots are harmonized per TB treatment outcome. $-\log_{10}$ -transformed p-values are plotted against $\log_2 FC$. Genes with $p < 0.05$ and $\log_2 FC < -0.6$ or > 0.6 were labelled as DEGs. (B) Heatmaps displaying $\log_2 FC$ of the DEGs and corresponding gene modules. The saturation of color represents the magnitude of differential expression. Differences were significant by means of linear mixed models. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

such as Sex, Age, HbA1c levels, and BMI did not have influence on the gene expression perturbation (Supplementary Figures 11-14). Next, we investigated whether accuracy could be improved by predicting TB treatment outcome after initiation of TB treatment, thus measuring the early treatment response. We identified a 22-gene signature to predict TB treatment outcome at two weeks after initiation of TB treatment (Table 3). The performance of the week two signature in predicting TB treatment outcome was slightly improved (pooled cohorts, AUC=0.834; Indonesia, AUC= 0.803; South Africa, AUC=0.867; TB-DM, AUC=0.92) compared to the diagnosis signature, especially in patients from the Indonesian cohort (AUC=0.867 versus AUC=0.744 at diagnosis). Furthermore, we identified a 14-gene month two signature, however this demonstrated a slightly lower accuracy in predicting TB treatment outcome compared to the diagnosis and week two gene signatures (pooled cohorts, AUC=0.791; Indonesia, AUC= 0.789; South Africa, AUC=0.805), but not in TB-DM (AUC=0.875). No significant differences were detected between models, comparing week two versus diagnosis, month two versus diagnosis, and month two versus week two using the DeLong test for correlated ROC curves (all TB groups with no discrimination between DM conditions: p-value=0.7986, 0.7271, 0.5626, respectively; TB-DM: p-value=0.3434, 0.5353, 0.6553).

Since we detected differences in the kinetics of gene expression of patients who had a good TB treatment outcome versus patients with a poor TB treatment outcome (Figure 1), we next assessed whether a “delta” gene signature, by subtracting week two values from diagnosis, could improve the predictive performance. The delta signature encompassed seven genes (*GNLY*, *MRC1*, *GBP5*, *NLRP1*, *FLCN1*, *ZNF532*, and *IFIT2*) and slightly improved predictive performance (pooled cohorts, AUC=0.849; South Africa, AUC=0.839 and Indonesia, AUC=0.872) compared to the week two and diagnosis signatures (Supplementary Figure S15, Supplementary Table S8). Multiple genes were included in more than one gene signature (Supplementary Figure S16A), of which four genes (*GBP1*, *GBP5*, *FCGR1A*, *INDO*) are shown in Figure 4B. Next, we validated performance of the diagnosis signature and month two signature on an independent Indian validation cohort (28), which like our cohorts, included diabetic patients. Our diagnosis gene signature had high predictive power on the Indian validation cohort (AUC=0.749) (Figure 4C). The week two and delta signatures could not be validated on the Indian cohort, because samples were not collected two weeks after initiation of TB treatment in this cohort. Importantly, three genes (*CD3E*, *PTPRCv1*, *NOD2*) that were included in our gene signatures, were also part of gene signatures described by Sivakumaran et al. (Supplementary Figure S16B). Finally, we assessed whether gene signatures with improved performance could be obtained by applying SMOTE (49) as an alternative sampling technique. A diagnosis SMOTE gene signature was obtained that

TABLE 3 | dcRT-MLPA treatment outcome signatures obtained by pooling the study groups and the cohorts.**Diagnosis Signature**

Gene	Module
GBP1	IFN signaling genes
FCGR1A	IFN signaling genes
STAT1	IFN signaling genes
IFITM3	IFN signaling genes
BCL2	Apoptosis - Survival
CCL4	Treg associated genes
TLR9	Pattern recognition receptors
CD274	IFN signaling genes

Week 2 Signature

Gene	Module
GBP5	IFN signaling genes
INDO	IFN signaling genes
GBP1	IFN signaling genes
BMP6	Cell growth - proliferation
CXCL9	Chemokines
GATA3	Th2 associated genes
FCGR1A	IFN signaling genes
MMP9	Inflammation
PTPRCv1	T cell subset markers
SPP1	Inflammation
CD3E	T cell subset markers
ASAP1	Small GTPases - (Rho) GTPase activating proteins
IL5	Th2 associated genes
TNFRSF1B	Apoptosis - Survival
NLRP2	Inflammasome components
MRC1	Pattern recognition receptors
NLRP6	Inflammasome components
IL22RA1	Th17 associated genes
VEGF	Cell growth - proliferation
KIF1B	Intracellular transport
CCL19	Chemokines
CD209	Pattern recognition receptors

Month 2 Signature

Gene	Module
BLR1	G protein-coupled receptors
BMP6	Cell growth - proliferation
CCL13	Chemokines
GBP1	IFN signaling genes
GBP2	IFN signaling genes
GBP5	IFN signaling genes
IFI16	IFN signaling genes
IL9	Th9 associated genes
INDO	IFN signaling genes
MMP9	Inflammation
NOD2	Pattern recognition receptors
OAS3	IFN signaling genes
PTPRCv2	T cell subset markers
TAP1	IFN signaling genes

Genes that appeared in more than one gene signature (diagnosis, week two or month two) are shown in bold.

Gene signatures were obtained by down-sampling the majority class (good treatment outcome).

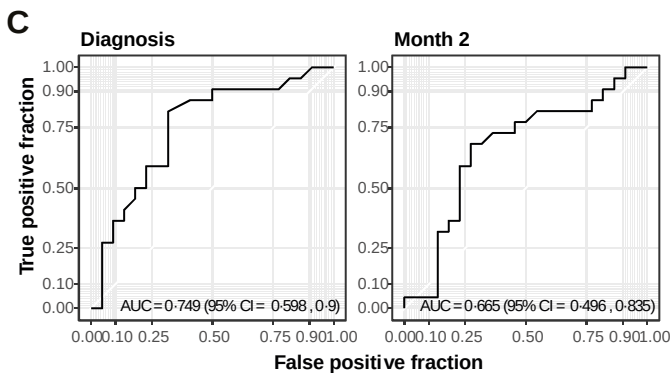
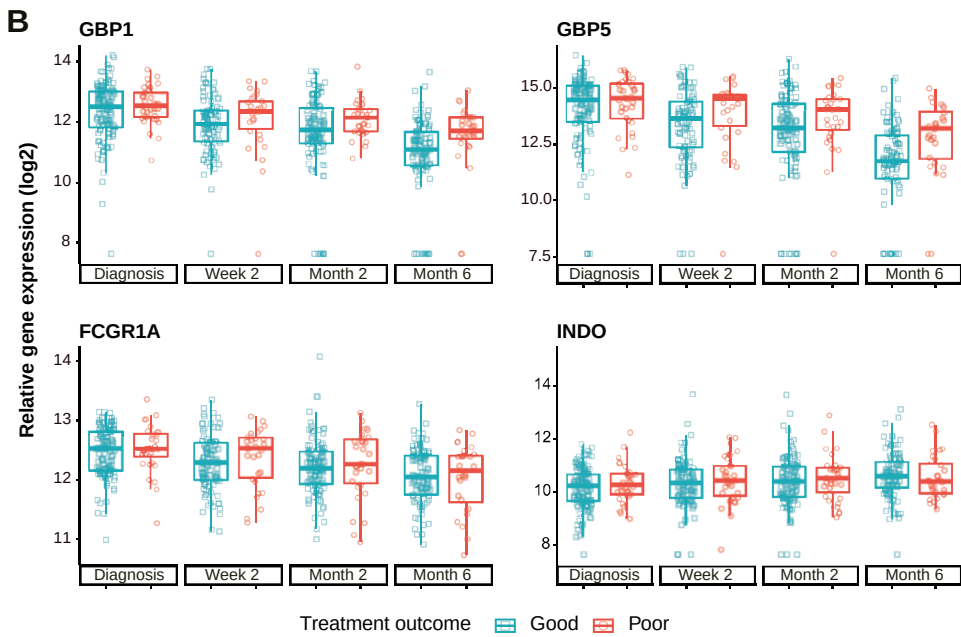
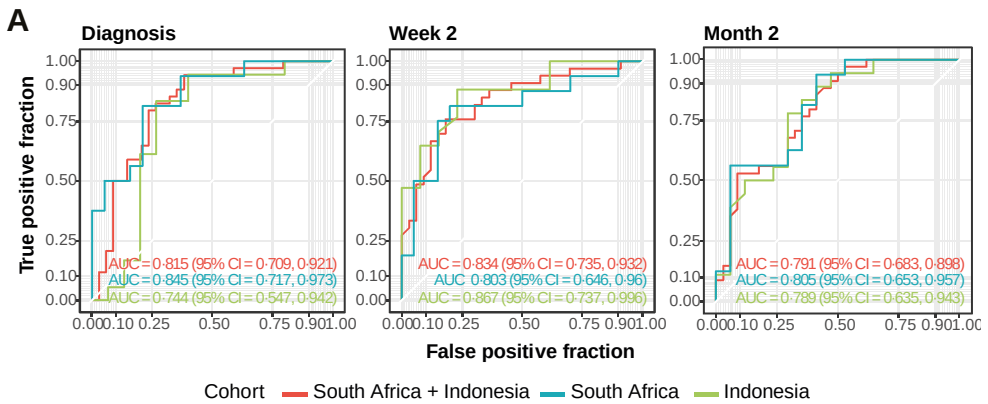


FIGURE 4 | Prediction of TB treatment outcome in dcRT-MLPA data from peripheral blood. (A) ROC curves showing the predictive power of the gene signatures identified in the balanced pooled cohort (South Africa and Indonesia) to classify TB patients at diagnosis (left panel), two weeks (middle panel) or two months (right panel) after initiation of TB treatment into patients who had a good TB treatment outcome and patients who had a poor TB treatment outcome, using the RFE-RF model and LOOCV. The dataset was balanced by down-sampling to encompass the same number of individuals with poor and good TB treatment outcome (diagnosis, $n=34$; week two, $n=33$; month two, $n=34$). (B) Gene expression kinetics of the single genes encompassing the diagnosis or week two gene signatures predicting TB treatment outcome in the pooled cohort. Box plots depict GAPDH-normalized, log₂-transformed median gene expression values and the IQR, while the whiskers represent the data within the $Q1-1.5 \times IQR$ and $Q3+1.5 \times IQR$ interval. (C). ROC curves showing the predictive power of the gene signatures identified in the balanced pooled cohort (South Africa and Indonesia) to classify TB patients from an external validation cohort (India) at diagnosis (left panel) or two months (right panel) after initiation of TB treatment into patients who had a good TB treatment outcome and patients who had a poor TB treatment outcome. The dataset was balanced by down-sampling to encompass the same number of individuals with poor and good TB treatment outcome (diagnosis, $n=22$; month two, $n=22$). Abbreviations: AUC, area under the curve; CI, confidence interval.

showed overlap with the diagnosis gene signatures obtained by random down-sampling (Supplementary Table S9, Supplementary Figure S16C). The SMOTE signature produced a high degree of accuracy in discriminating patients with a good TB treatment outcome from patients with a poor TB treatment outcome, but performed with lower accuracy compared to the diagnosis signature obtained by random down-sampling (pooled cohorts, $AUC=0.728$; South Africa, $AUC=0.695$; Indonesia, $AUC=0.765$) (Supplementary Figure S17). The diagnosis SMOTE signature exerted a similar predictive capacity on the external Indian cohort compared to the down-sampling signature (SMOTE, $AUC=0.704$; down-sampling, $AUC=0.749$).

Taken together, we identified gene signatures with high predictive power on TB treatment outcome, irrespective of DM as comorbidity, in patients from South Africa and Indonesia and in patients from the external Indian validation cohort.

Discussion

In this study, we identified peripheral blood transcriptional signatures which predict TB treatment success and failure in a TB cohort with patients with varying hyperglycaemia or DM. Previous studies developing biomarker signatures of TB treatment success, recurrence or failure (24-26, 56) did not include people with DM comorbidity, and we have previously found that concomitant DM impairs existing TB diagnosis signature accuracy (32). Here we showed DM also affects existing TB treatment-response biomarker signatures in the RNA-Seq dataset, suggesting that they should be derived with cohorts including this population, and our data could be used to test validity of other putative biomarker signatures, such as the RESPONSE5 signature (25), in this population.

Our whole cohort dataset, from which we generated TB treatment outcome signatures, was derived using our dcRT-MLPA gene set, which did not contain most of the genes reported in previous signatures, except *GBP5*, which was included in our week two and month two gene signatures. Sivakumaran et al. (28) recently reported baseline and month two gene signatures predicting TB treatment outcome at six months after initiation of TB treatment, using the same material (whole blood), technique (dcRT-MLPA) and gene set. Notably, our TB treatment outcome gene signatures showed some overlap with the signatures reported by Sivakumaran et al. (*CD3E*, *PTPRCv1*, *NOD2*), suggesting that these genes are useful in predicting TB treatment outcome independently of ethnic background. Furthermore, our TB treatment outcome gene signatures showed overlap of genes of the TB risk signature predicting TB progression from healthy controls more than a year before onset of TB (*GBP1*, *GBP2*, *GBP5*, *FCGR1A*, *STAT1*, *TAP1*) (22). Within our study, 12 genes (*BCL2*, *BMP6*, *CCL13*, *CD209*, *FCGR1A*, *GBP1*, *GBP5*, *INDO*, *MMP9*, *MRC1*, *STAT1*, *TLR9*) were overlapping between gene signatures, including both the gene signatures obtained by down-sampling and the gene signatures obtained by SMOTE. The occurrence of genes in multiple gene signatures within this study and between studies highlights the power of transcriptomic biomarkers in predicting TB treatment outcome and suggests that universal biomarkers can be applied to cohorts of different ethnicity and independently of the DM/glycaemia status of TB patients.

Patients with a poor TB treatment outcome responded to TB treatment at the level of individual genes, as detected by downregulation of genes (*GBP1*, *GBP2*, *GBP5*, *IFITM3*) that have been associated with active TB and upregulation of genes (*CD3E*, *PTPRCv1*, *NLRP1*, *GNLY*, *PRF1*, *BCL2*) that show lower expression in patients with active TB compared to LTBI or healthy controls (15, 20, 39, 55). However, MDP analysis showed that the response to TB treatment was diminished in those with a poor TB treatment outcome compared to patients who had a good TB treatment outcome. Notably, the majority of genes that were significantly downregulated in patients who had a good TB treatment outcome, but not in patients who had a poor TB treatment outcome, are involved in IFN signaling (*IRF7*, *IFIT2*, *IFIT3*, *STAT2*, *IFI6*, *TAP2*). This suggests that a poor TB treatment outcome was reflected by persisting IFN signaling response and supports a role for type I IFN signaling in TB pathogenesis (15, 57).

TAGAP was significantly increased in patients who had a poor TB treatment outcome in the pooled South African and Indonesian cohort as well as in both cohorts separately. *TAGAP* encodes T-cell activation Rho-GTPase-activating protein, however, the exact role of *TAGAP* in *Mtb* pathogenesis is currently unknown. Several studies have linked *TAGAP* with active TB; *TAGAP* was enriched for differential acetylation peaks upon *Mtb* infection in granulocytes (58) and *TAGAP* was induced upon vaccination with AERAS-402 vaccine

encoding a fusion protein of *Mtb* antigens (59). Furthermore, *TAGAP* had higher expression in TB patients compared to LTBI and healthy controls (55) and, surprisingly, lower expression in pulmonary TB compared to household controls (60). Our data showing that *TAGAP* expression was significantly increased during TB treatment in patients who had a poor TB treatment outcome could indicate that *TAGAP* is actively involved in TB pathogenesis or that *TAGAP* expression is a consequence of persisting *Mtb* infection, potentially by enhanced T-cell activation, but this remains to be investigated.

There are several limitations of the current study. First, the sample size in this study was not based on an *a priori* power calculation, as this study was part of a larger study investigating differences in gene expression in patients with varying degrees of hyperglycemia. To increase statistical power, we therefore pooled patients from two cohorts (South Africa and Indonesia), which introduced heterogeneity within the studied groups. However, this can also be a strength, potentially increasing application over different ethnic backgrounds. Second, the low sample size ($n=15$) of patients with a poor TB treatment outcome with or without DM (Table 1) reduced the robustness of the identified signatures in a DM-stratified analysis in our prediction model, which is a limitation of this study. TB-DM patients received different DM medications and considering the low sample size, we were unable to correct for this, which could have been a confounding factor. However, we have previously seen similar changes in blood gene expression in people with pre-existing DM taking medication and with transient hyperglycaemia not receiving medication, indicating this has minimal effect (32). We have also previously found that metformin has minimal impact on circulating blood transcriptomes (61), as do TB drugs (62). Our study was also too small to perform a stratified analysis based on the type of poor outcome, *i.e.* death, default, treatment failure or recurrence after treatment completion. We could not determine whether recurrence was due to relapse or reinfection: relapse and failure both occur when insufficient mycobacteria have been killed to permit immunological control. In future, our model should be tested prospectively in a large TB treatment cohort, to determine its validity for recurrence/relapse as well as failure. Third, there were missing values in the cohort study. The missing values occurred as a result of random drop-outs or technical errors caused by low quantity or quality of some samples, and therefore the use of linear mixed models for the DEA was employed, as it most likely produced unbiased results. Fourth, although the prevalence of hyperglycaemia/DM is not indicated in the majority of other TB biomarker studies, which is a limitation of these studies considering the rising incidence of TB-DM comorbidity, our study contained many patients with high HbA_{1c} levels. Although this may have introduced a bias, the strength of this approach is that TB treatment outcome signatures have been developed that can be applied to patients independently of their glycaemia/DM status.

Furthermore, we showed that our eight-gene diagnosis signature had a high performance (AUC=0.749) when tested on an external validation cohort in patients with a different ethnic background (India), which is striking since geographic or ethnic variations may significantly impact on the immune responses to TB.

In this study, we demonstrated the potential of gene signatures to predict TB treatment outcome, in a cohort including patients with concomitant DM or hyperglycaemia. Here, we have focused on the host transcriptome, but in later development stages, host and clinical host factors, such as extent of lung cavitation at diagnosis and through treatment, can be added to improve prediction accuracy, as was suggested and demonstrated by Sivakumaran et al. (28). Identification of a diagnosis gene signature containing only eight genes in this study, and even fewer genes in signatures reported by others (26, 27), indicates that clinically-implementable biomarker signatures can be developed using transcriptomic-based approaches using easily accessible whole blood, and that are promising as surrogate marker for sputum culture conversion.

Contributors

Study concept and design: BA, RvC, RR, PCH, GW, SAJ, JAC, MCH, HMD, THMO, JMC; Patient recruitment, Sample collection, processing and selection: BA, KR, STM, LK, PCH, KS, RR, GW; Clinical database design, curation, maintenance: SK-B, JAC; Laboratory Experiments and data acquisition: CE, SvV, JSL, JMC; Data Analysis and interpretation: CLrvD, CE, SvV, VK, SAJ, CW, MCH, HMD, THMO, EV, JMC; Writing the manuscript: CLrvD, CE, SAJ, HMD, THMO, EV, JMC; Critical Revision of the manuscript: CLrvD, CE, KR, STM, SAJ, PCH, JAC, MCH, HMD, THMO, EV, JMC; All authors read and approved the final version of the manuscript.

Declaration of Interests

GW had patents to methods of tuberculosis diagnosis and to tuberculosis biomarkers unrelated to the current study. The rest of the authors declare no financial or commercial conflicts of interest.

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Data Sharing Statement

RNA sequence data have been submitted to NCBI Gene Expression Omnibus (GEO) under accession number GSE193979. dcRT-MLPA data can be found in Supplementary Table S6.

References

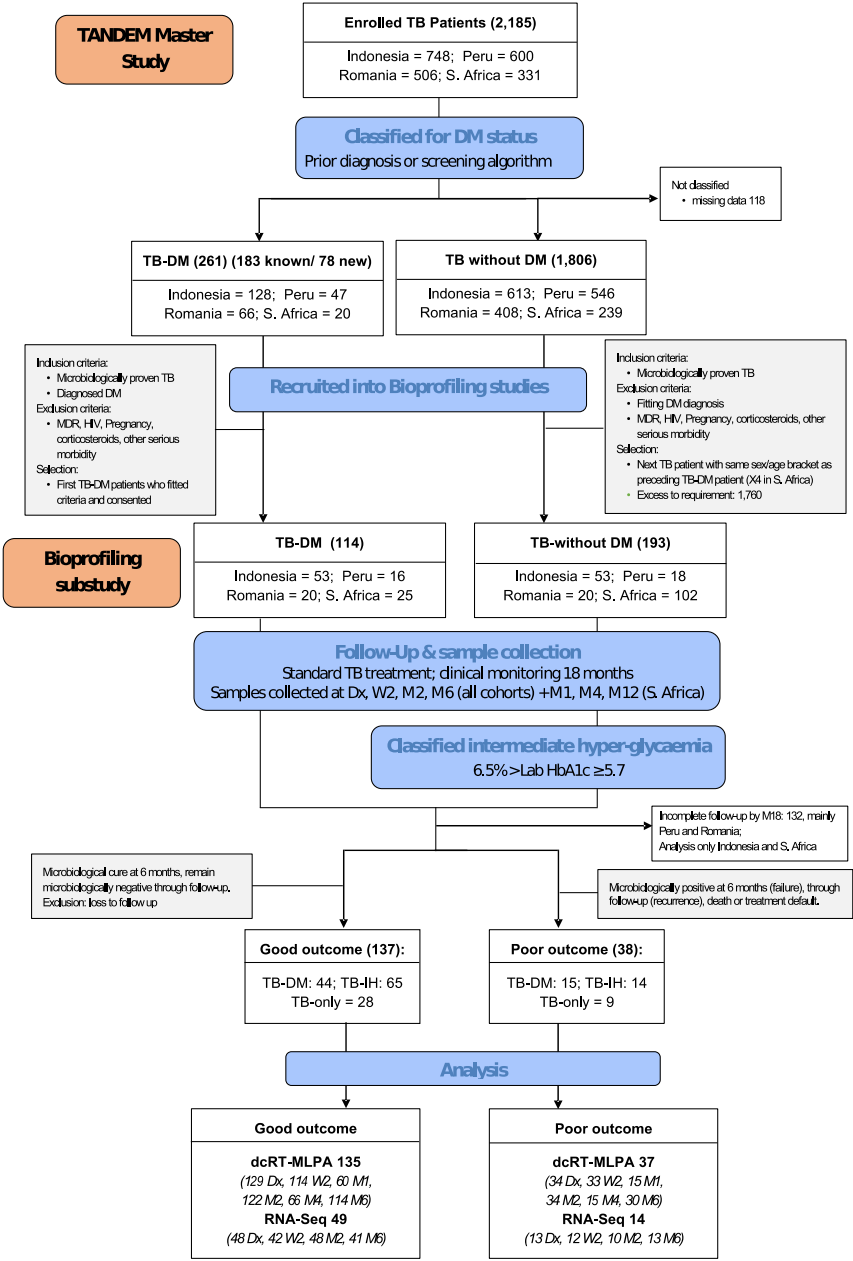
1. World Health Organization. Global tuberculosis report 2021. Geneva: World Health Organization. Licence: CC BY-NC-SA 3.0 IGO., 2021.
2. Alemu MA, Yesuf A, Girma F, et al. Impact of HIV-AIDS on tuberculosis treatment outcome in Southern Ethiopia—A retrospective cohort study. *Journal of Clinical Tuberculosis and Other Mycobacterial Diseases* 2021; 25: 100279.
3. Huangfu P, Ugarte-Gil C, Golub J, Pearson F, Critchley J. The effects of diabetes on tuberculosis treatment outcomes: an updated systematic review and meta-analysis. *Int J Tuberc Lung Dis* 2019; 23(7): 783-96.
4. Jeon CY, Murray MB. Diabetes mellitus increases the risk of active tuberculosis: a systematic review of 13 observational studies. *PLoS med* 2008; 5(7): e152.
5. Baker MA, Harries AD, Jeon CY, et al. The impact of diabetes on tuberculosis treatment outcomes: a systematic review. *BMC medicine* 2011; 9(1): 1-15.
6. Dooley KE, Tang T, Golub JE, Dorman SE, Cronin W. Impact of diabetes mellitus on treatment outcomes of patients with active tuberculosis. *The American journal of tropical medicine hygiene* 2009; 80(4): 634.
7. Alisjahbana B, Sahiratmadja E, Nelwan EJ, et al. The effect of type 2 diabetes mellitus on the presentation and treatment response of pulmonary tuberculosis. *Clinical Infectious Diseases* 2007; 45(4): 428-35.
8. Tuomilehto J. The emerging global epidemic of type 1 diabetes. *Current diabetes reports* 2013; 13(6): 795-804.
9. Saeedi P, Petersohn I, Salpea P, et al. Global and regional diabetes prevalence estimates for 2019 and projections for 2030 and 2045: Results from the International Diabetes Federation Diabetes Atlas. *Diabetes research and clinical practice* 2019; 157: 107843.
10. Horne DJ, Royce SE, Gooze L, et al. Sputum monitoring during tuberculosis treatment for predicting outcome: systematic review and meta-analysis. *The Lancet infectious diseases* 2010; 10(6): 387-94.
11. Bhat J, Rao V, Muniyandi M, Yadav R, Karforma C, Luke C. Impact of sputum quality and quantity on smear and culture positivity: findings from a tuberculosis prevalence study in central India. *Transactions of the Royal Society of Tropical Medicine Hygiene* 2014; 108(1): 55-6.
12. Hernández P, Punchak M, Camacho M, Hepple P, McNerney R. Investigating the quality of expectorated sputum for tuberculosis diagnosis in Bolivia. *The International Journal of Tuberculosis and Lung Disease* 2015; 19(9): 1065-7.
13. Meyer AJ, Atuheire C, Worodria W, et al. Sputum quality and diagnostic performance of GeneXpert MTB/RIF among smear-negative adults with presumed tuberculosis in Uganda. *PLoS One* 2017; 12(7): e0180572.
14. Warsinske H, Vashisht R, Khatri P. Host-response-based gene signatures for tuberculosis diagnosis: A systematic comparison of 16 signatures. *PLoS medicine* 2019; 16(4): e1002786.
15. Berry MP, Graham CM, McNab FW, et al. An interferon-inducible neutrophil-driven blood transcriptional signature in human tuberculosis. *Nature* 2010; 466(7309): 973-7.
16. Bloom CI, Graham CM, Berry MP, et al. Detectable changes in the blood transcriptome are present after two weeks of antituberculosis therapy. *PloS one* 2012; 7(10): e46191.
17. Cliff JM, Lee JS, Constantinou N, et al. Distinct phases of blood gene expression pattern through

- tuberculosis treatment reflect modulation of the humoral immune response. *J Infect Dis* 2013; 207(1): 18-29.
18. Kaforou M, Wright VJ, Oni T, et al. Detection of tuberculosis in HIV-infected and-uninfected African adults using whole blood RNA expression signatures: a case-control study. *PLoS Med* 2013; 10(10): e1001538.
 19. Maertzdorf J, McEwen G, Weiner 3rd J, et al. Concise gene signature for point-of-care classification of tuberculosis. *EMBO molecular medicine* 2016; 8(2): 86-95.
 20. Sweeney TE, Braviak L, Tato CM, Khatri P. Genome-wide expression for diagnosis of pulmonary tuberculosis: a multicohort analysis. *The Lancet Respiratory Medicine* 2016; 4(3): 213-24.
 21. Scriba TJ, Penn-Nicholson A, Shankar S, et al. Sequential inflammatory processes define human progression from *M. tuberculosis* infection to tuberculosis disease. *PLoS Pathog* 2017; 13(11): e1006687.
 22. Zak DE, Penn-Nicholson A, Scriba TJ, et al. A blood RNA signature for tuberculosis disease risk: a prospective cohort study. *The Lancet* 2016; 387(10035): 2312-22.
 23. Mistry R, Cliff JM, Clayton CL, et al. Gene-expression patterns in whole blood identify subjects at risk for recurrent tuberculosis. *J Infect Dis* 2007; 195(3): 357-65.
 24. Cliff JM, Cho J-E, Lee J-S, et al. Excessive cytolytic responses predict tuberculosis relapse after apparently successful treatment. *The Journal of infectious diseases* 2016; 213(3): 485-95.
 25. Thompson EG, Du Y, Malherbe ST, et al. Host blood RNA signatures predict the outcome of tuberculosis treatment. *Tuberculosis* 2017; 107: 48-58.
 26. Warsinske HC, Rao AM, Moreira FM, et al. Assessment of validity of a blood-based 3-gene signature score for progression and diagnosis of tuberculosis, disease severity, and treatment response. *JAMA network open* 2018; 1(6): e183779-e.
 27. Suliman S, Thompson EG, Sutherland J, et al. Four-gene pan-African blood signature predicts progression to tuberculosis. *American journal of respiratory and critical care medicine* 2018; 197(9): 1198-208.
 28. Sivakumaran D, Jennum S, Vaz M, et al. Combining host-derived biomarkers with patient characteristics improves signature performance in predicting tuberculosis treatment outcomes. *Communications Biology* 2020; 3(1): 359.
 29. Ugarte-Gil C, Alisjahbana B, Ronacher K, et al. Diabetes mellitus among pulmonary tuberculosis patients from 4 tuberculosis-endemic countries: the TANDEM study. *Clinical Infectious Diseases* 2020; 70(5): 780-8.
 30. Viswanathan V, Kumpatla S, Aravindalochanan V, et al. Prevalence of diabetes and pre-diabetes and associated risk factors among tuberculosis patients in India. *PLoS One* 2012; 7(7): e41367.
 31. Restrepo BI, Camerlin AJ, Rahbar MH, et al. Cross-sectional assessment reveals high diabetes prevalence among newly-diagnosed tuberculosis cases. *Bulletin of the World Health Organization* 2011; 89: 352-9.
 32. Eckold C, Kumar V, Weiner J, et al. Impact of Intermediate Hyperglycemia and Diabetes on Immune Dysfunction in Tuberculosis. *Clinical Infectious Diseases* 2021; 72(1): 69-78.
 33. Prada-Medina CA, Fukutani KF, Kumar NP, et al. Systems immunology of diabetes-tuberculosis comorbidity reveals signatures of disease complications. *Scientific reports* 2017; 7(1): 1-16.
 34. Ruslami R, Koesoemadinata RC, Soetedjo NNM, et al. The effect of a structured clinical algorithm on glycemic control in patients with combined tuberculosis and diabetes in Indonesia: A randomized trial. *Diabetes Research and Clinical Practice* 2021; 173: 108701.
 35. Dobin A, Davis CA, Schlesinger F, et al. STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* 2013; 29(1): 15-21.
 36. Andrews S. FastQC: a quality control tool for high throughput sequence data. 2010. <http://www.bioinformatics.babraham.ac.uk/projects/fastqc2020>.
 37. Anders S, Pyl PT, Huber W. HTSeq—a Python framework to work with high-throughput sequencing data. *bioinformatics* 2015; 31(2): 166-9.

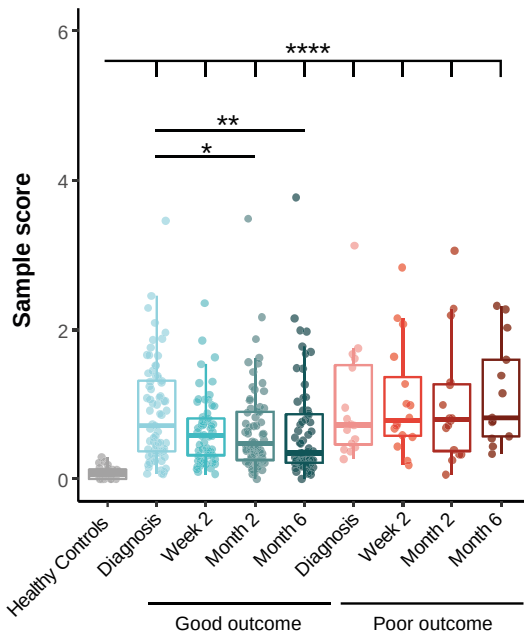
38. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome biology* 2014; 15(12): 1-21.
39. Joosten S, Goeman J, Sutherland J, et al. Identification of biomarkers for tuberculosis disease using a novel dual-color RT–MLPA assay. *Genes & Immunity* 2012; 13(1): 71-82.
40. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal statistical society: series B (Methodological)* 1995; 57(1): 289-300.
41. Lever M RP, Nakaya H. mdp: Molecular Degree of Perturbation calculates scores for transcriptome data samples based on their perturbation from controls. R package version 1.12.0. 2021. <https://mdp.sysbio.tools/>.
42. Chikina M, Zaslavsky E, Sealfon SC. CellCODE: a robust latent variable approach to differential expression analysis for heterogeneous cell populations. *Bioinformatics* 2015; 31(10): 1584-91.
43. Abbas AR, Wolslegel K, Seshasayee D, Modrusan Z, Clark HF. Deconvolution of blood microarray data identifies cellular activation patterns in systemic lupus erythematosus. *PloS one* 2009; 4(7): e6098.
44. Novershtern N, Subramanian A, Lawton LN, et al. Densely interconnected transcriptional circuits control cell states in human hematopoiesis. *Cell* 2011; 144(2): 296-309.
45. Weiner 3rd J, Domaszewska T. tmod: an R package for general and multivariate enrichment analysis. *PeerJ Preprints* 2016; 4: e2420v1.
46. Nueda MJ, Tarazona S, Conesa A. Next maSigPro: updating maSigPro bioconductor package for RNA-seq time series. *Bioinformatics* 2014; 30(18): 2598-602.
47. Bates D, Mächler M, Bolker B, Walker S. Fitting linear mixed-effects models using lme4. *arXiv preprint arXiv:14065823* 2014.
48. Gregorutti B, Michel B, Saint-Pierre P. Correlation and variable importance in random forests. *Statistics and Computing* 2017; 27(3): 659-78.
49. Chawla NV, Bowyer KW, Hall LO, Kegelmeyer WP. SMOTE: synthetic minority over-sampling technique. *Journal of artificial intelligence research* 2002; 16: 321-57.
50. Liaw A, Wiener M. Classification and regression by randomForest. *R news* 2002; 2(3): 18-22.
51. Kuhn M. Building predictive models in R using the caret package. *Journal of statistical software* 2008; 28(1): 1-26.
52. Raudvere U, Kolberg L, Kuzmin I, et al. g:Profiler: a web server for functional enrichment analysis and conversions of gene lists (2019 update). *Nucleic Acids Research* 2019; 47(W1): W191-W8.
53. Huang DW, Sherman BT, Lempicki RA. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. *Nucleic acids research* 2009; 37(1): 1-13.
54. Gebremicael G, Kassa D, Quinten E, et al. Host gene expression kinetics during treatment of tuberculosis in HIV-coinfected individuals is independent of highly active antiretroviral therapy. *The Journal of infectious diseases* 2018; 218(11): 1833-46.
55. Gebremicael G, Kassa D, Alemayehu Y, et al. Gene expression profiles classifying clinical stages of tuberculosis and monitoring treatment responses in Ethiopian HIV-negative and HIV-positive cohorts. *PLoS One* 2019; 14(12): e0226137.
56. Heyckendorf J, Marwitz S, Reimann M, et al. Prediction of anti-tuberculosis treatment duration based on a 22-gene transcriptomic model. *European respiratory journal* 2021.
57. Moreira-Teixeira L, Mayer-Barber K, Sher A, O'Garra AJJoEM. Type I interferons in tuberculosis: Foe and occasionally friend. *J Journal of Experimental Medicine* 2018; 215(5): 1273-85.
58. del Rosario RCH, Poschmann J, Kumar P, et al. Histone acetylome-wide association study of tuberculosis. *bioRxiv* 2019: 644112.
59. Sivakumaran D, Blatner G, Bakken R, et al. A 2-Dose AERAS-402 Regimen Boosts CD8+ Polyfunctionality in HIV-Negative, BCG-Vaccinated Recipients. *Frontiers in Immunology* 2021; 12: 2141.
60. Sivakumaran D, Ritz C, Gjøen JE, et al. Host blood RNA transcript and protein signatures for

- sputum-independent diagnostics of tuberculosis in adults. *Frontiers in immunology* 2020; 11: 3795.
61. Lachmandas E, Eckold C, Bohme J, Koeken V, Marzuki MB, Blok B, et al. Metformin Alters Human Host Responses to *Mycobacterium tuberculosis* in Healthy Subjects. *J Infect Dis.* 2019;220(1):139-50.
 62. Broderick C, Cliff JM, Lee JS, Kaforou M, Moore DA. Host transcriptional response to TB preventive therapy differentiates two sub-groups of IGRA-positive individuals. *Tuberculosis (Edinb).* 2021;127:102033.

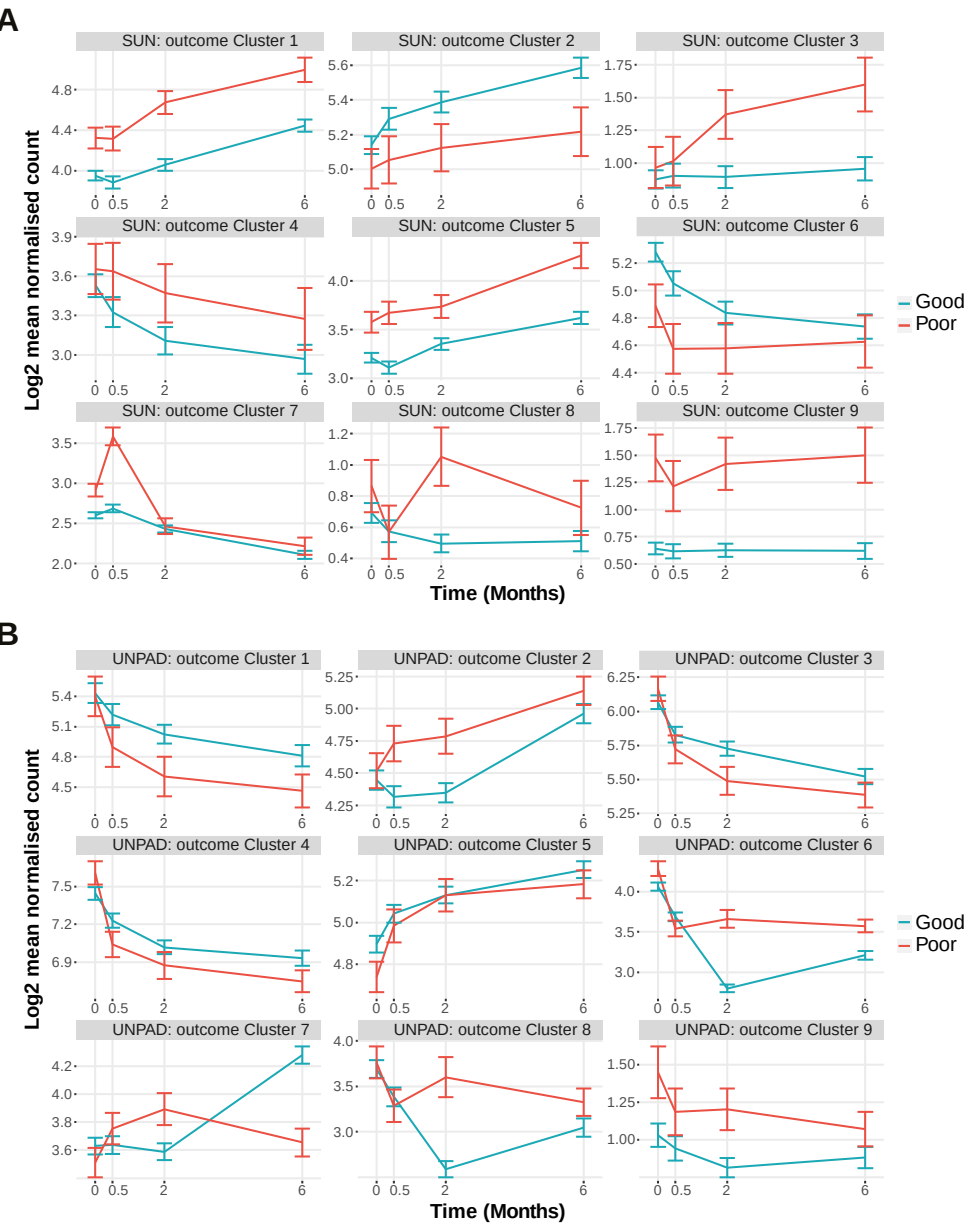
Supplementary Figures



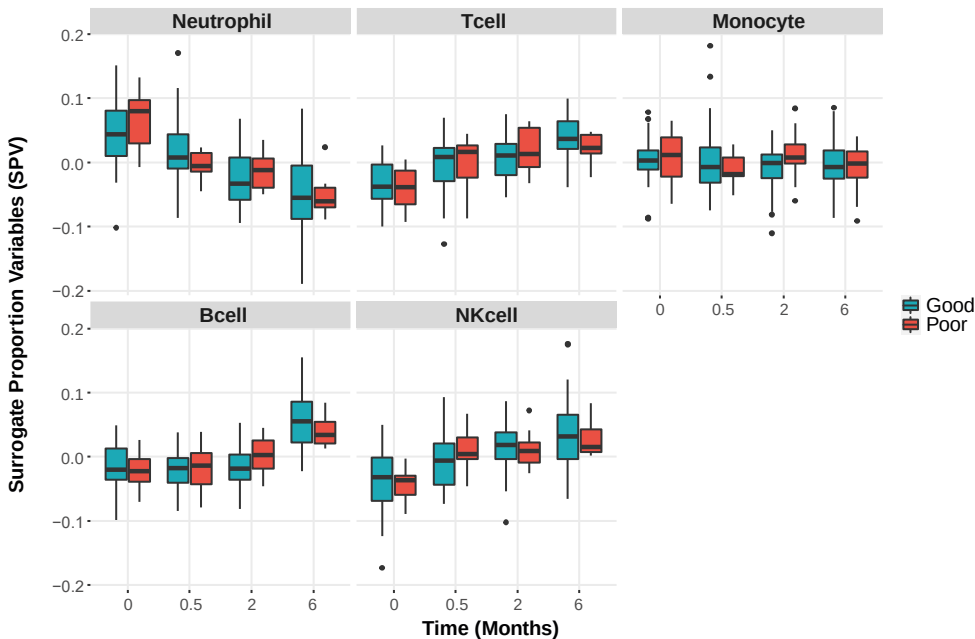
SUPPLEMENTARY FIGURE S1 | Overview of participant enrolment in the TANDEM study, classification and inclusion in the longitudinal gene expression analysis. Dx: diagnosis; M: month; W: week



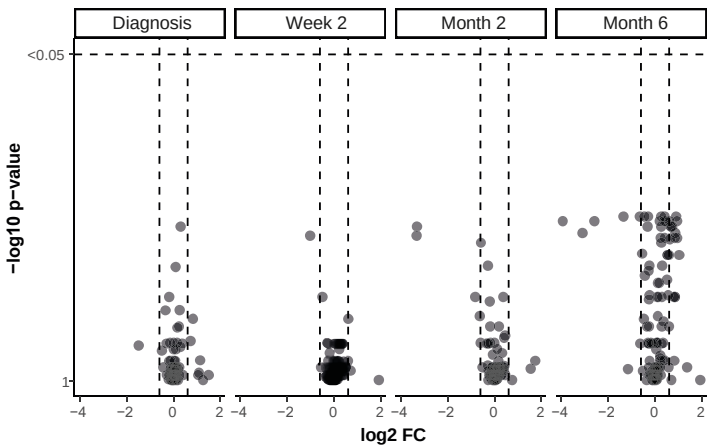
SUPPLEMENTARY FIGURE S2 | MDP plot representing the change in gene expression perturbation in TB patients from South Africa categorized based on treatment outcome. Blood transcriptomes from TB patients who had a good or poor treatment outcome were determined by dcRT-MLPA. The extent of overall difference in gene expression, relative to the median of expression in healthy controls, was calculated for individual patients at the timepoints shown. The bars and whiskers show the median and data within the Q1-1.5 x inter quartile range (IQR) and Q3+1.5 x IQR interval. Differences were significant by Mann-Whitney U-test with Benjamini-Hochberg correction for multiple testing. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.



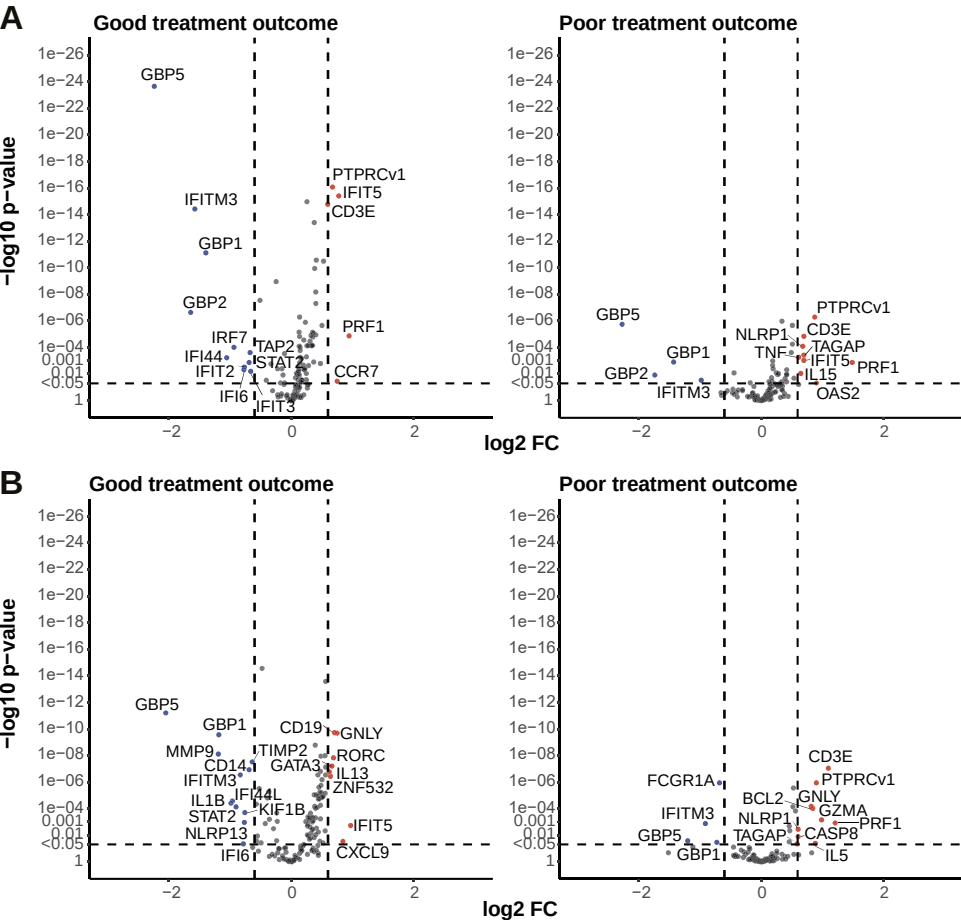
SUPPLEMENTARY FIGURE S3 | Differential change in gene expression in TB patients through treatment in those who had a good or poor treatment outcome. MaSigPro analysis was conducted on the blood RNA-Seq data from TB patients from South Africa (A) or Indonesia (B), to identify genes which were significantly differentially expressed between those patients with a good or poor outcome. Plots show hierarchical clusters of genes, and bars show mean $\pm$ 1 SEM. Data were filtered to remove lowly abundant transcripts prior to analysis.

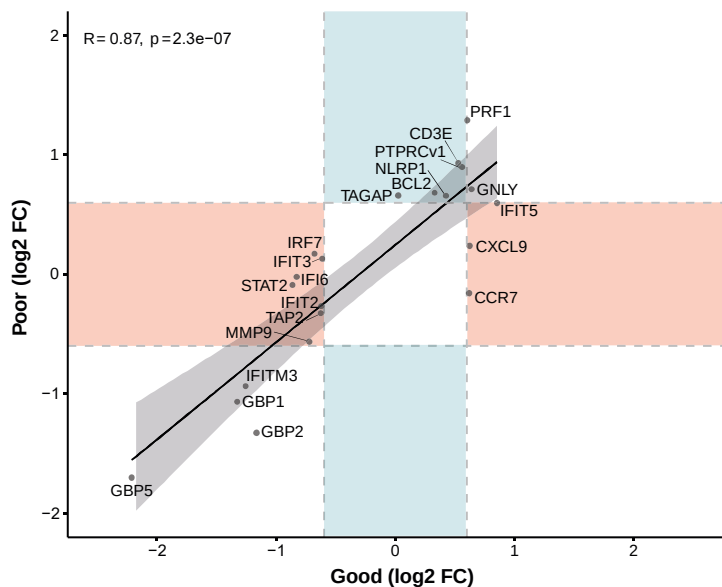


SUPPLEMENTARY FIGURE S4 | Cell population estimates in good and poor treatment outcomes in South Africa and Indonesia. Estimates of relative differences in cell proportions were calculated from RNA-seq data using R package CellCODE. IRIS and DMAP data sets used as reference. Bars and whiskers show median and 1.5 x IQR. There were no significant differences identified between Good and Poor TB treatment outcome groups at any timepoint for any cell type.

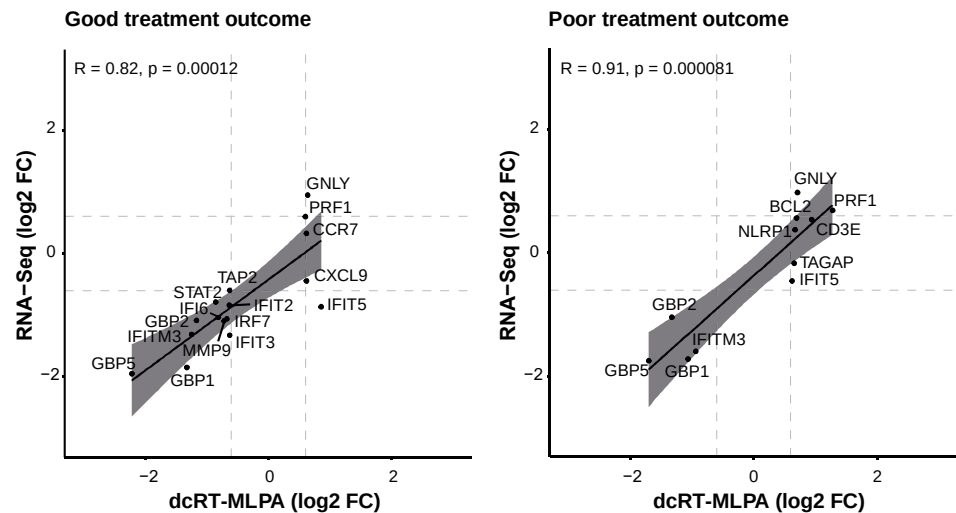


SUPPLEMENTARY FIGURE S5 | Differential expression analysis in patients from South Africa and Indonesia who had a poor treatment outcome versus patients who had a good treatment outcome at the indicated timepoints. Non-parametric Mann-Whitney U-test with Benjamini-Hochberg correction for multiple testing was applied to test for statistical differences between the groups.

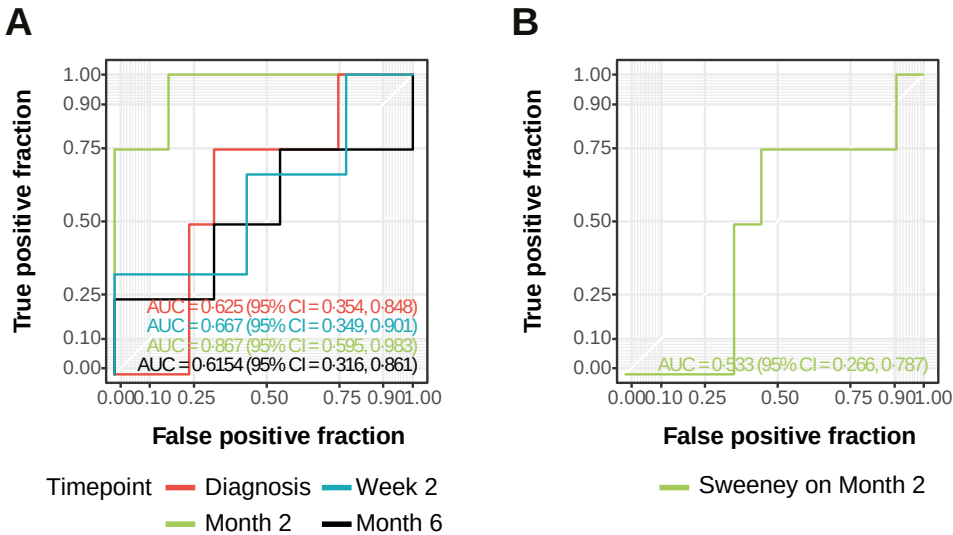




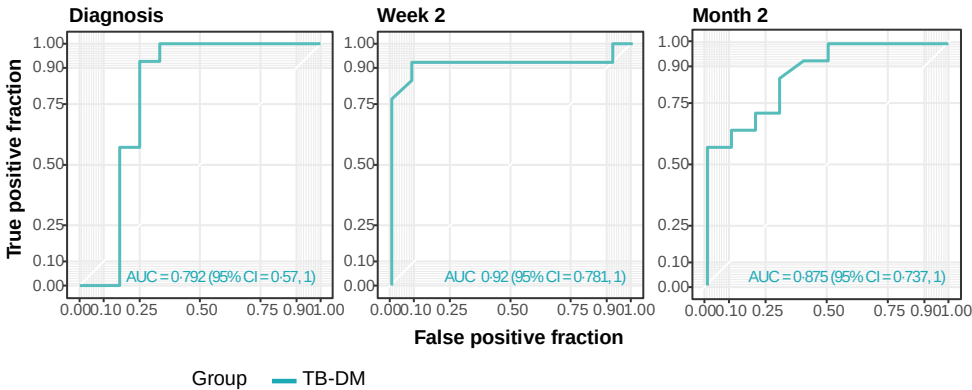
SUPPLEMENTARY FIGURE S7 | Scatter plot representing Pearson correlations between the longitudinal DEGs in TB patients who had a poor treatment outcome versus the longitudinal DEGs in TB patients who had a good treatment outcome. Values are plotted as log₂ FC (month 6-diagnosis). Black line corresponds to line of best fit and shaded bands indicate confidence intervals. Red shaded areas indicate genes that were identified as DEGs only in patients who had a good treatment outcome and blue shaded areas indicate genes that were identified as DEGs only in patients who had a poor treatment outcome.



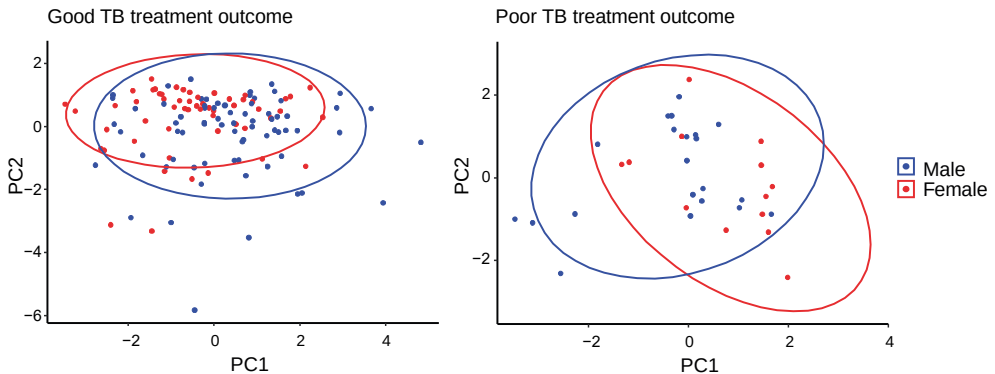
SUPPLEMENTARY FIGURE S8 | Scatter plots representing Pearson correlations between the longitudinal DEGs identified by dcRT-MLPA versus the same genes identified by RNA-Seq. Values are plotted as log₂ FC (month six-diagnosis). Black line corresponds to line of best fit and shaded bands indicate confidence intervals.



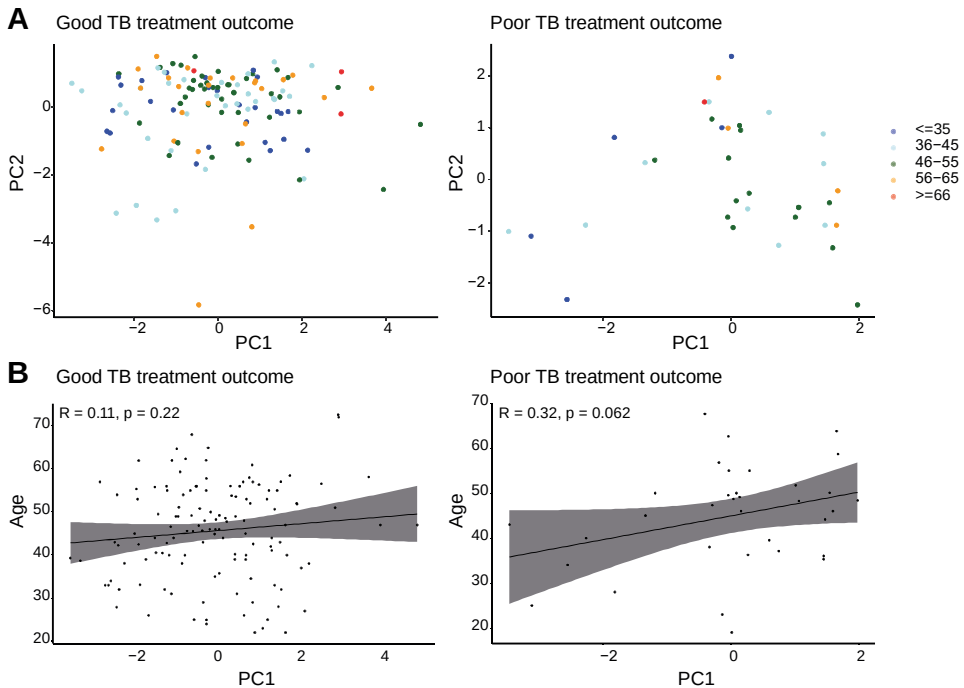
SUPPLEMENTARY FIGURE S9 | Prediction of treatment outcome in RNA-Seq data from peripheral blood. ROC curves showing the predictive power of (A) the gene signatures identified in the pooled cohort (South Africa and Indonesia) or (B) the Sweeney gene signature to classify TB patients at the indicated timepoints after initiation of TB treatment into patients who had a good treatment outcome and patients who had a poor treatment outcome. Data were split into training and test sets (60/40). For each time point a gene signature was generated by RFE and a weighted model fitted using glmnet method. Weights of $1/\text{frequency} * 0.5$ were used. Abbreviations: AUC, area under the curve; CI, confidence interval.



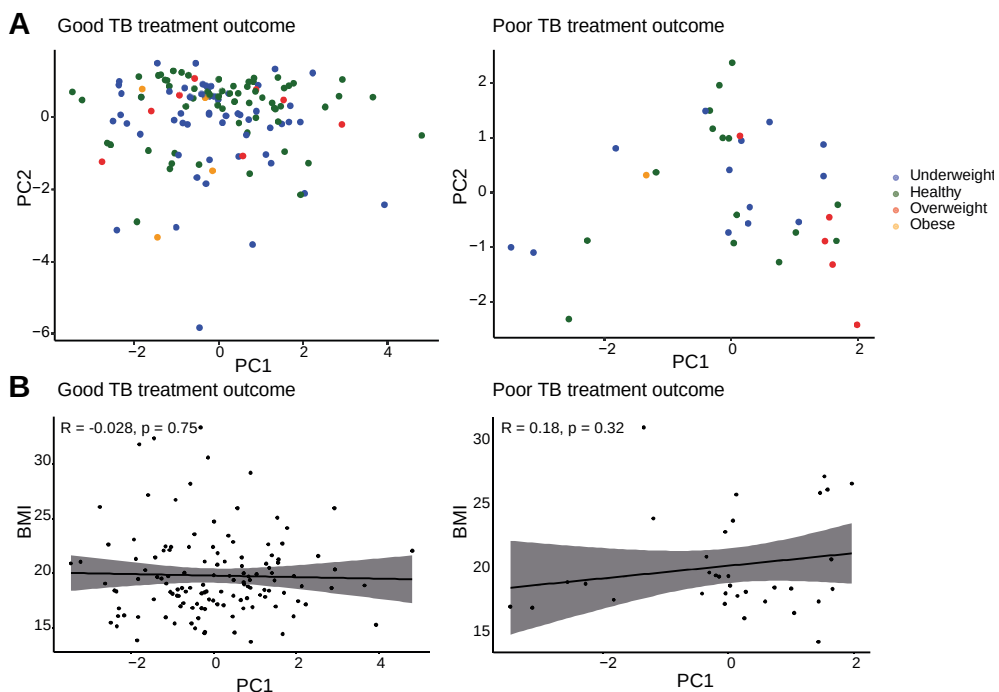
SUPPLEMENTARY FIGURE S10 | Performance evaluation of the gene signatures identified without discriminating between DM conditions in predicting the outcome of TB treatment in participants with TB and diabetes. ROC curve showing the predictive power of the gene signature identified in the balanced pooled cohort without discriminating between DM conditions to classify TB patients with diabetes into patients who had a good treatment outcome and patients who had a poor treatment outcome, using the RFE - RF model and LOOCV. The dataset was balanced by down-sampling to encompass the same number of individuals with poor and good treatment outcome. Abbreviations: AUC, area under the curve; CI, confidence interval.



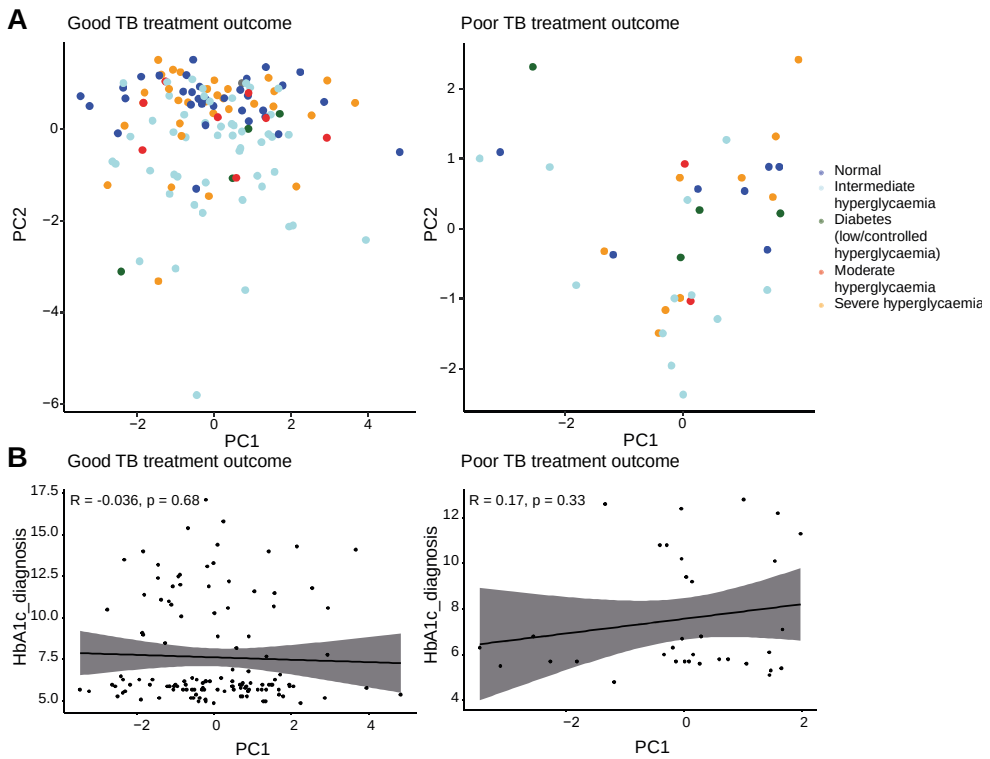
SUPPLEMENTARY FIGURE S11 | PCA analysis of Sex of TB patients in South Africa and Indonesia. TB patients are classified into good TB treatment outcome (left panel) and poor TB treatment outcome (right panel) at diagnosis. Abbreviations: PC, principal component.



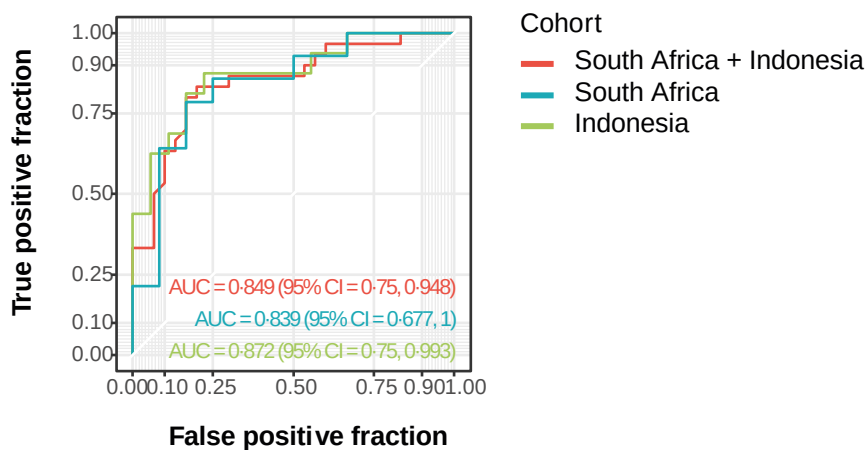
SUPPLEMENTARY FIGURE S12 | PCA analysis of Age of TB patients in South Africa and Indonesia. Plots showing (A) PC2 versus PC1 or (B) the correlation between Age and PC1 in TB patients that are classified into good TB treatment outcome (left panels) and poor TB treatment outcome (right panels) at diagnosis. Abbreviations: PC, principal component



SUPPLEMENTARY FIGURE S13 | PCA analysis of BMI of TB patients in South Africa and Indonesia. Plots showing (A) PC2 versus PC1 or (B) the correlation between BMI and PC1 in TB patients that are classified into good TB treatment outcome (left panels) and poor TB treatment outcome (right panels) at diagnosis. Abbreviations: PC, principal component.

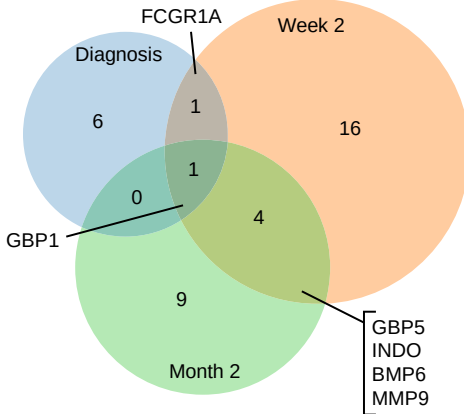


SUPPLEMENTARY FIGURE S14 | PCA analysis of HbA1c levels at diagnosis of TB patients in South Africa and Indonesia. Plots showing (a) PC2 versus PC1 or (b) the correlation between HbA1c and PC1 in TB patients that are classified into good TB treatment outcome (left panels) and poor TB treatment outcome (right panels) at diagnosis. Abbreviations: PC, principal component.

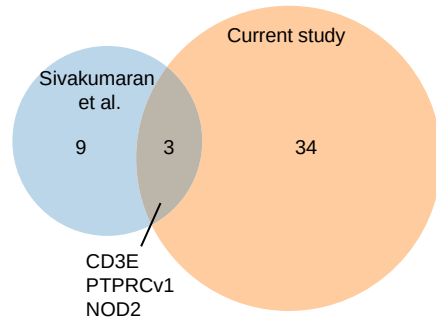


SUPPLEMENTARY FIGURE S15 | Identification of a delta (week 2 minus diagnosis) signature predicting the outcome of TB treatment. ROC curve showing the predictive power of the gene signature identified in the balanced pooled cohort to classify TB patients into patients who had a good treatment outcome and patients who had a poor treatment outcome, using the RFE - RF model and LOOCV. The dataset was balanced by down-sampling to encompass the same number of individuals with poor and good treatment outcome. Abbreviations: AUC, area under the curve; CI, confidence interval.

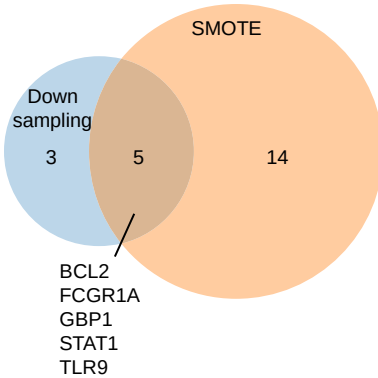
A Venn diagram - within cohort



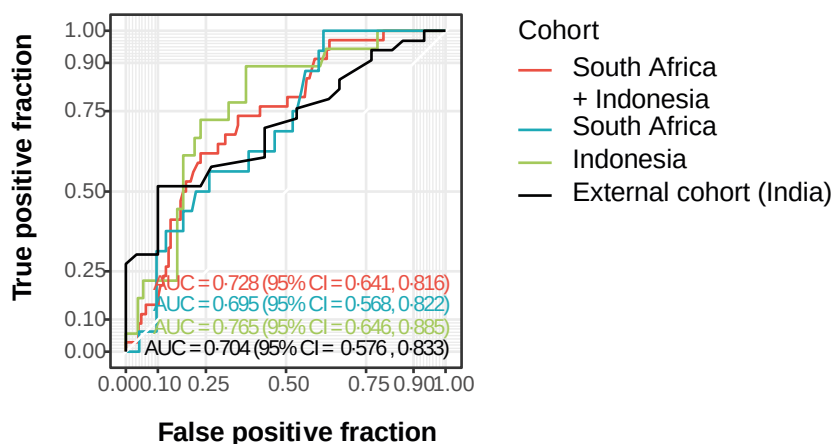
B Venn diagram - between cohorts



C Venn diagram - sampling methods at diagnosis



SUPPLEMENTARY FIGURE S16 | Venn diagrams showing the number of genes encompassing treatment outcome signatures as identified by RFE-RF models. (A) Venn diagram displaying the number of unique and overlapping genes comparing diagnosis (blue), week two (orange) and month two (green) gene signatures obtained by random-downsampling. (B) Venn diagram displaying the number of unique and overlapping genes comparing the gene signatures in the current study (diagnosis, week two and month two; orange) obtained by random-downsampling with the gene signatures published by Sivakumaran et al. (blue). (C) Venn diagram displaying the number of unique and overlapping genes comparing the diagnosis gene signature obtained by random-downsampling (blue) with the diagnosis gene signature obtained by applying SMOTE sampling (orange) to balance the classes. Overlapping genes are annotated.



SUPPLEMENTARY FIGURE S17 | Identification of a SMOTE diagnosis signature predicting the outcome of TB treatment. ROC curves showing the predictive power of the gene signatures identified in the balanced pooled cohort (South Africa and Indonesia) and validated in the South African and Indonesian cohort or in an external Indian cohort. TB patients are classified into patients who had a good treatment outcome and patients who had a poor treatment outcome at diagnosis, using the RFE - RF model and LOOCV. The dataset was balanced by SMOTE to encompass the same number of individuals with poor and good treatment outcome. Abbreviations: AUC, area under the curve; CI, confidence interval.

Supplementary Tables

SUPPLEMENTARY TABLES S1-S6 can be downloaded at:

[https://www.thelancet.com/journals/ebiom/article/PIIS2352-3964\(22\)00354-1/fulltext#supplementaryMaterial](https://www.thelancet.com/journals/ebiom/article/PIIS2352-3964(22)00354-1/fulltext#supplementaryMaterial)

SUPPLEMENTARY TABLE S7 | Gene signatures for each timepoint - RNA-Seq.

<u>Diagnosis</u>	<u>Week 2</u>	<u>Month 2</u>	<u>Month 6</u>
MADCAM1	FGFR3	CD79B	DNMT3B
MTFR2	ICAM3	PIK3CB	HAUS4
TM4SF1	SLC7A4	MEF2BNB-MEF2B	SLC22A18
CLCN1	PACSLN2	TCL1A	RTN4
WASF4P	HPN	TANGO6	CCRL2
ANKDD1B	ERGIC1	PTGES3	SERPINE2
HIST1H2BO	IL1R2	DOCK7	LYSMD2
TCTEX1D2	SSPN	ZNHIT6	WASH2P
RPSAP54	DOK4	KIF14	IL17RC
WASH4P	PRKCG	PARD6B	ARFIP1
AC026185.1	TCN1	E2F5	CCDC68
RPS2P44	GCM1	AK7	PPP1R14A
RP11-466P24.6	GAS2L3	MPZL2	RIMBP3
RP11-1105G2.4	SYT2	AKR1E2	Y_RNA
RP11-229P13.25	NR1I2	OXSRI	MIR140
MIR3136	TENM4	CNIH2	LEPROT
CTB-50L17.9	SLC45A3	ACBD7	RPL18P10
RP11-272L13.4	WNK2	TNF	AC099552.2
RP11-92K15.3	DPCD	PTGES3L	RP11-544A12.5
RP11-305L7.7	CXCL11	SPIB	FLT1P1
	B3GALNT1	USP32P1	RP11-295K2.3
	SLC22A1	IGHD	RP11-56M3.1
	HIST1H2AJ	GOLGA2P7	AC132186.1
	SNRNP35	AC096579.7	C1orf213
	CLCN1	RP11-458F8.1	UGDH-AS1
	CTD-3088G3.8	AC002543.2	RP11-175P13.3
	SULF2	IGKV1D-16	RP11-638I2.10
	CLEC4C	LINC00617	RP11-705O1.8
	RNU6-1079P	RP11-407G23.4	SEN3-EIF4A1
	AGAP7	RP11-588K22.2	RP11-401F2.3
	STARD7-AS1	RP11-325K4.2	RP11-1109F11.3
	YBX1P1	AL133153.1	U91328.22
	PRRT4	RP11-479F13.1	
	LINC01001		
	BCAS2P2		
	RP11-459O1.2		
	AL627309.1		
	IFNG-AS1		
	CTD-3051D23.4		
	RP4-647C14.3		
	CTD-2006K23.1		
	RP11-457M11.5		
	BX649553.4		
	CTB-39G8.3		
	MUC8		
	AC092299.8		

SUPPLEMENTARY TABLE S8 | Delta signature Good vs Poor obtained by pooling the study groups and the cohorts.**Delta (Week two - Diagnosis) Signature**

Gene	Module
GNLY	Cytotoxicity markers
MRC1	Pattern recognition receptors
GBP5	IFN signaling genes
NLRP1	Inflammasome components
FLCN1	Apoptosis - Survival
ZNF532	Transcriptional regulators - activators
IFIT2	IFN signaling genes

Genes that appeared also in the diagnosis or week two gene signatures are shown in bold. Gene signatures were obtained by downsampling the majority class (Good treatment outcome).

SUPPLEMENTARY TABLE S9 | SMOTE signature Good vs Poor obtained by pooling the study groups and the cohorts.**Diagnosis Signature**

Gene	Module
AREG	Cell growth - proliferation
BCL2	Apoptosis - Survival
CASP8	Apoptosis - Survival
CCL13	Chemokines
CD209	Pattern recognition receptors
CLEC7A	Pattern recognition receptors
CTLA4	Treg associated genes
CX3CL1	Chemokines
FCGR1A	IFN signaling genes
GBP1	IFN signaling genes
IL12B	Myeloid associated genes
LTF	Anti-microbial activity
NLRP3	Inflammasome components
STAT1	IFN signaling genes
TIMP2	Inflammation
TLR8	Pattern recognition receptors
TLR9	Pattern recognition receptors
TNFRSF1A	Apoptosis - Survival
ZNF331	Transcriptional regulators - activators

Gene signature obtained by applying SMOTE sampling technique. Genes that are also identified in the Diagnosis signature obtained by downsampling are shown in bold.

Supplementary Methods

Reverse-Transcriptase Multiplex Ligation-dependent Probe Amplification (dcRT-MLPA)

For each target-specific sequence, a specific RT primer was designed located immediately downstream of the left- and right-hand half-probe target sequence. 125 ng RNA was reverse transcribed to cDNA by incubation at 37°C for 15 min, using RT-primer mix and Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Promega, Leiden, The Netherlands). Reverse transcriptase was inactivated by heating at 98°C for two minutes. The left- and right-hand half probes were hybridized to the cDNA at 60°C overnight and annealed half-probes were ligated at 54°C for 15 minutes using ligase-65 (MRC-Holland). Ligase-65 was subsequently inactivated by heating at 98°C for five minutes. Ligated probes were amplified by PCR: 33 cycles at 95°C for 30 seconds, 58°C for 30 seconds and 72°C for 60 seconds, followed by one cycle at 72°C for 20 minutes. PCR products were 1:10 diluted in Highly deionized (Hi-Di) formamide (ThermoFisher) containing 400HD Rhodamine X (ROX) fluorophore size standard (ThermoFisher). PCR products were denatured at 95°C for five minutes, stored immediately at 4°C and analyzed on an Applied Biosystems 3730 capillary sequencer in GeneScan mode (BaseClear, Leiden, The Netherlands). Trace data were analyzed using GeneMapper software 5 (Applied Biosystems, Warrington, UK). The areas of each assigned peak (arbitrary units) were exported for analysis in R (version 3.6.3). Data were corrected for batch effect and normalized to housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). Signals below the threshold value for noise cutoff in GeneMapper (log2 transformed peak area 7.64) were assigned the threshold value for noise cutoff.

RT primers and half-probes were designed by Leiden University Medical Centre (LUMC, Leiden, The Netherlands) and comprised sequences for four housekeeping genes and 144 selected key immune-related genes to profile the following compartments of the human immune response (Supplementary Table S1): (1) Adaptive immune responses: T-cell responses; Th1 responses; Th2 responses; Th17/22 responses; Treg responses; T-cell cytotoxicity; Immune cell subset markers including B-cells and NK-cells. (2) Innate immune responses: Myeloid-associated markers and scavenger receptors; Pattern recognition receptors; Inflammasome components. (3) Inflammatory and IFN-signalling genes. (4) Other genes: Anti-microbial activity; Apoptosis/cell survival; E3 ubiquitin protein ligases; Small GTPases/(Rho)GTPase activating proteins; Additional chemokines; Cell growth/proliferation; Cell activation; Transcriptional regulators/activators; Intracellular transport; Mitochondrial Stress/Proteasome; Inflammation.

Linear Mixed Models for identification of DEGs

Longitudinal DEGs were identified by means of linear mixed models using the *lmer* function of the *lme4* package in R (1). To increase statistical power, datasets of all TB patients included in the South African and Indonesian cohorts were pooled independent of diabetes/glycaemia status and split based on treatment outcome. Models were fitted on *GAPDH*-normalized log₂-transformed targeted gene expression data. Outcome-time interactions were included as fixed effects and the patients ID-time interactions were included as random effects.

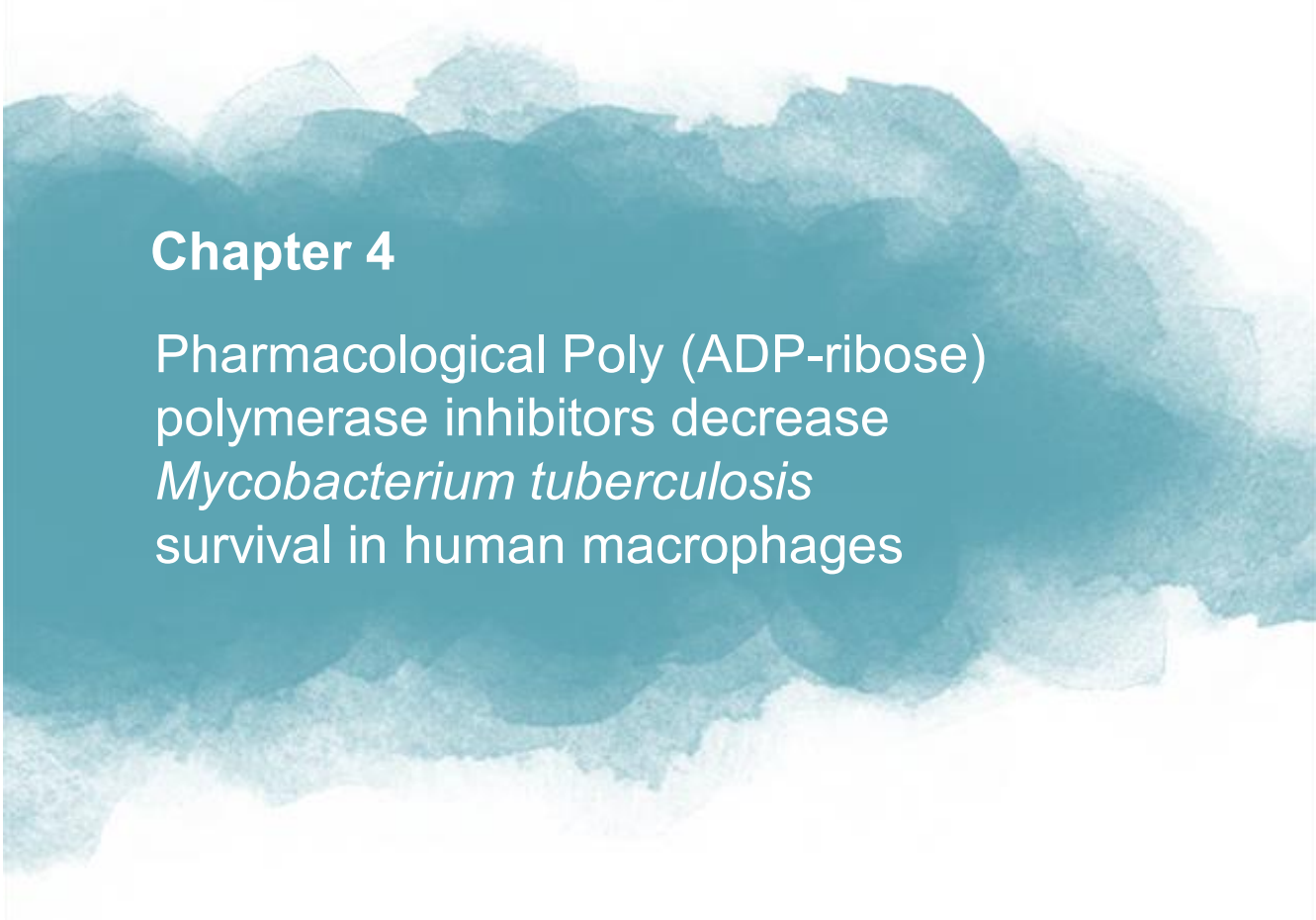
Identification of gene signatures for treatment outcome

For modelling analyses, RNA-Seq data were randomly split into training and test sets (60/40) using the R package *caret* (2). Feature selection was performed for each timepoint using RFE (3) with repeated cross validation as the re-sampling method (n=10). A weighted model was fitted using *glmnet* method, using weights 1/frequency * 0.5 and repeated cross validation for re-sampling (n=10). Each model was used to make predictions on the corresponding test set.

To identify signatures associated with treatment outcome in dcRT-MLPA data, TB patients of South Africa and Indonesia were pooled independent of diabetes/glycaemia status. To balance the dataset, we applied two random sampling approaches using R: (1) a down-sampling approach, reducing the number of patients in the majority class (i.e. good treatment outcome) and (2) an up-sampling approach by generating synthetic data from existing data using the SMOTE (4) function from the *DMwR* package in R, resulting in equal numbers of patients in both classes. Then, RFE (3), available in the *caret* R package (2), was applied with K-fold validation (K=10) to the entire data set search for the optimal combination and number of top-ranking genes able to separate TB patients with a good and poor treatment outcome. RFE is a powerful approach for variable selection in high-dimensional data by selecting features that fit a model and removing the weakest feature (or features) until the specified number of features is reached. Once the best predictors of treatment outcome were identified, the expression values of these genes were extracted from the dataset. We subsequently applied RF (5) as machine learning algorithm on the dataset and evaluated the performance by LOOCV, both available on the *caret* R package (2).

References

1. Bates D, Mächler M, Bolker B, Walker S. Fitting linear mixed-effects models using lme4. *arXiv preprint arXiv:1406.5823*. 2014.
2. Kuhn M. Building predictive models in R using the caret package. *Journal of statistical software*. 2008;28(1):1-26.
3. Gregorutti B, Michel B, Saint-Pierre P. Correlation and variable importance in random forests. *Statistics and Computing*. 2017;27(3):659-78.
4. Chawla NV, Bowyer KW, Hall LO, Kegelmeyer WP. SMOTE: synthetic minority over-sampling technique. *Journal of artificial intelligence research*. 2002;16:321-57.
5. Liaw A, Wiener M. Classification and regression by randomForest. *R news*. 2002;2(3):18-22.

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Chapter 4

Pharmacological Poly (ADP-ribose) polymerase inhibitors decrease *Mycobacterium tuberculosis* survival in human macrophages

Cassandra L.R. van Doorn, Sanne A.M. Steenbergen, Kimberley V. Walburg,
and Tom H.M. Ottenhoff

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Abstract

Diabetes mellitus (DM) is correlated with increased susceptibility to and disease progression of tuberculosis (TB), and strongly impairs effective global TB control measures. To better control the TB-DM co-epidemic, unravelling the bidirectional interactivity between DM-associated molecular processes and immune responses to *Mycobacterium tuberculosis* (*Mtb*) is urgently required. Since poly (ADP-ribose) polymerase (PARP) activation has been associated with DM and with *Mtb* infection in mouse models, we have investigated whether PARP inhibition by pharmacological compounds can interfere with host protection against *Mtb* in human macrophage subsets, the predominant target cell of *Mtb*. Pharmacological inhibition of PARP decreased intracellular *Mtb* and MDR-*Mtb* levels in human macrophages, identifying PARP as a potential target for host-directed therapy against *Mtb*. PARP inhibition was associated with modified chemokine secretion and upregulation of cell surface activation markers by human macrophages. Targeting LDH, a secondary target of the PARP inhibitor rucaparib, resulted in decreased intracellular *Mtb*, suggesting a metabolic role in rucaparib-induced control of *Mtb*. We conclude that pharmacological inhibition of PARP is a potential novel strategy in developing innovative host-directed therapies against intracellular bacterial infections.

Introduction

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis* (*Mtb*). Around 10 million new TB cases and 1.4 million deaths are reported annually and one quarter of the world's population is latently infected with *Mtb* (1). Different risk factors have been identified for progression of latent TB infection (LTBI) to active disease, including age, malnutrition, coinfection with human immunodeficiency virus (HIV) or cytomegalovirus (CMV), and diabetes mellitus (DM) (1–3). In addition, recent studies have shown that DM is also associated with poorer clinical TB outcome following anti-bacterial treatment. Current estimates are that 15% of the global better treatment of TB burden is now associated with DM (4, 5). Host-directed therapy (HDT) offers the potential for Multi-Drug-Resistant (MDR)-TB, as well as shorten current standard (6–9 month) treatment regimens, thereby reducing toxicity, enhancing treatment compliance and as a result reducing emergence of de novo drug resistance. To better control the TB-DM co-epidemic, unravelling the bidirectional interactions between DM-dysregulated molecular processes and immune responses to *Mtb* is essential.

Macrophages (MFs) are known for their dual role in TB pathogenesis, as they provide a primary host niche for *Mtb* during infection while also being key host effector cells eliminating *Mtb*, and therefore represent key target cells in developing and evaluating novel therapeutic strategies for TB/DM, including innovative HDT. MFs are classically subdivided into pro-inflammatory macrophages (M1) and anti-inflammatory macrophages (M2), representing the polar ends of the macrophage spectrum. Both M1 and M2 have been reported in tuberculous granulomas, with M2 predominating in granulomas from patients with active TB (6).

Poly (ADP-ribose) polymerase (PARP) activation has been associated with cancer, diabetes and endothelial dysfunction in experimental mouse models and in humans (7–10). PARP-deficient mice and mice treated with PARP inhibitor PJ34 were resistant to streptozocin-induced diabetes, and pharmacological inhibition of PARP has been proposed for the treatment of DM (11–13). Interestingly, in addition, PARP inhibitors have recently been proposed as HDT compounds for reducing TB-induced inflammation (14), thereby providing a potential mechanistic link between the TB-DM co-morbidity at the molecular level. Several PARP inhibitors (PARPi) have been clinically approved for the treatment of cancer or are currently being evaluated in clinical trials for the treatment of advanced BRCA1/2 mutant ovarian and breast cancers, which can accelerate translation to HDTs against *Mtb*. PARP encompasses a family of enzymes involved in different cellular processes such as control of genomic stability, programmed cell death and DNA repair. When assisting in the repair of single-strand DNA nicks, PARP enzymes bind to single-stranded DNA breaks (SSB) or double-stranded DNA breaks (DSB) to generate PAR

polymers on itself (auto-PARylation), histones and chromatin-associated proteins, which together leads to chromatin relaxation and recruitment of repair proteins. Inhibition of PARP results in accumulation of SSB due to delayed DNA repair. Given the dual role of PARP activation on diabetes and tuberculosis infection, we hypothesized that small molecule inhibitors that interfere with PARP activation at the cellular level may be able to redirect macrophage function in response to *Mtb* infection. Such chemical compounds could be valuable tools as part of immunomodulatory HDT regimens.

In the current study, we explore the potential of PARP inhibitors in the treatment of intracellular *Mtb* infections in human macrophages. We demonstrate that pharmacological inhibition of PARP decreases intracellular *Mtb* in human macrophages and modulates the immune response of *Mtb*-infected human macrophages. To our knowledge, the potential of targeting PARP for HDT in a human model of *Mtb*-infection has not been reported yet, and our work identifies PARPi as novel HDT compounds against *Mtb* and possibly other intracellular infectious diseases.

Results

Pharmacological Inhibition of PARP Reduces *Mtb* Survival in Human Macrophages in a Host-Directed Manner

To study whether PARP may play a role during *Mtb* infections in human macrophages, we first evaluated intracellular *Mtb* survival after treatment with four clinically relevant PARPi (Figures 1A, B). We selected 10 μ M as a relatively high PARPi concentration, which has been shown to target PARP *in vitro* (15–17) and which allowed us to directly compare drug efficacy of PARPi with each other and with control kinase inhibitor H-89 (18, 19). Rucaparib significantly decreased intracellular *Mtb* CFUs in M1 and M2 more efficiently than H-89 (Figure 1C, $n=6$). Additionally, niraparib and pamiparib decreased intracellular *Mtb* in M1 and A-966492 decreased intracellular *Mtb* in M2, suggesting cell-specific effects of PARP inhibition on bacterial load for these compounds. Furthermore, we assessed the effect of PARPi on cytotoxicity using uninfected macrophages from four different donors, to ascertain that PARPi was not detrimental to healthy human macrophages. PARPi did not affect host-cell integrity and was not cytotoxic (Figure 1D). Next, we used a flow cytometry-based method (18) to evaluate whether the inhibition of *Mtb* outgrowth by PARPi was accompanied by a decrease in the percentage of cells harboring Venus-expressing *Mtb*. Rucaparib, but not other PARPi, significantly reduced the percentage of *Mtb*-infected cells both in M1 and M2 (Figure 1E, $n=8$), further corroborating the *Mtb*-inhibiting effect of rucaparib in human macrophages. To exclude that PARPi had direct microbicidal effects, liquid *Mtb* cultures were treated with PARPi in the absence of macrophages in two independent experiments (Figure 1F). None of the PARPi limited

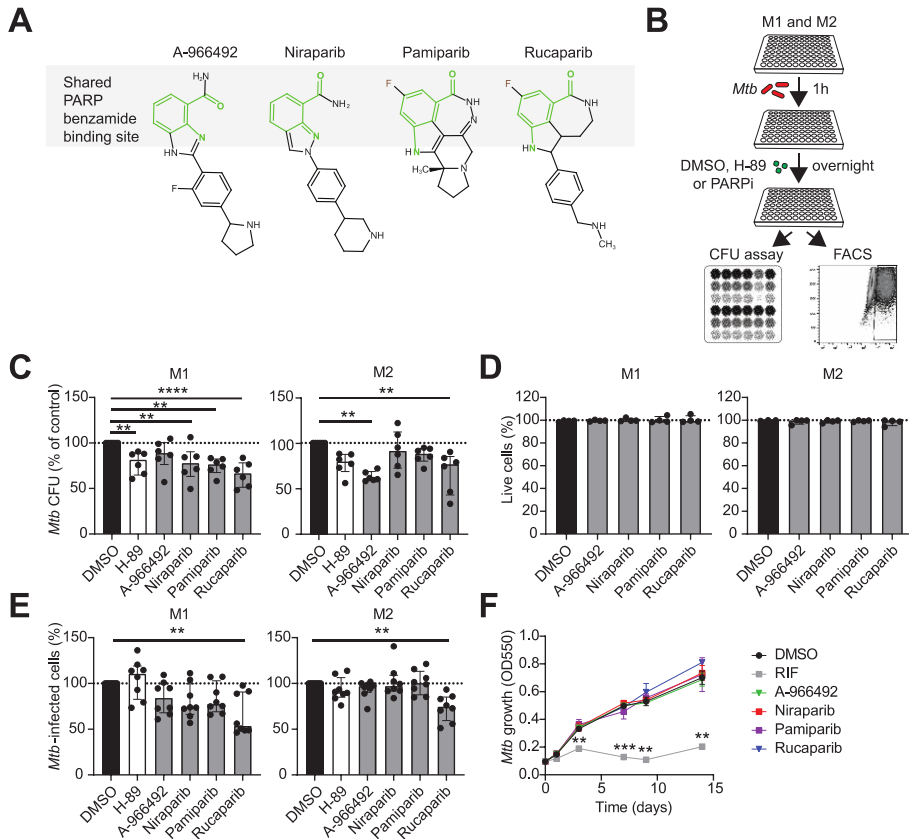


FIGURE 1 | Identification of PARP as potential host target during *Mtb* infections in human macrophages. (A) Chemical structures of the PARPi used in this study. The benzamide moiety that is shared between all PARP inhibitor structures is highlighted in light green. (B) Schematic representation of the experimental setup used in C and E. (C) *Mtb* H37Rv-infected M1 and M2 were treated overnight with PARPi (10 μ M), H-89 (10 μ M) or an equal volume of vehicle control DMSO (0.1% v/v). CFU data represent the median $\pm$ interquartile range of six different donors. Dots represent the mean from triplicate wells of a single donor. CFUs are expressed as percentage of vehicle control (i.e. DMSO). Differences were significant by RM one-way ANOVA with Dunnett's multiple test correction against DMSO. (D) Percentage of live M1 and M2 (i.e. PI-negative cells) after overnight treatment with PARPi (10 μ M) or an equal volume of vehicle control DMSO (0.1% v/v). Data represent the median $\pm$ interquartile range from four different donors. Differences were tested by Friedman's test with Dunn's multiple test correction against DMSO. (E) Percentage of M1 and M2 infected with Venus-expressing *Mtb* H37Rv that were treated as in C. Data represent the median $\pm$ interquartile range from eight different donors. Differences were significant by Friedman's test with Dunn's multiple test correction against DMSO. (F) Liquid *Mtb* H37Rv growth (in the absence of cells) was monitored for 14 days after addition of positive control RIF (20 μ g/ml), PARPi (10 μ M) or vehicle control DMSO (0.1% v/v). Data represent the means $\pm$ S.D. of triplicate wells from a representative experiment out of two independent experiments. Differences were significant by RM two-way ANOVA with Dunnett's multiple test correction against DMSO. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

bacterial growth directly, indicating that PARPi restrict intracellular outgrowth of *Mtb* by modulating host pathways.

To investigate whether PARPi-induced inhibition of intracellular bacterial growth was specific for *Mtb*, we evaluated whether pharmacological inhibition of PARP also restricted growth of other intracellular pathogenic bacteria, including *Mycobacterium avium* (*Mav*), *Salmonella enterica* serovar Typhimurium (*Stm*) and methicillin-resistant *Staphylococcus aureus* (MRSA). Interestingly, niraparib, but not other PARPi, significantly decreased *Stm* CFUs in M1 and M2 (Figure 2A, n=6) without exhibiting direct microbicidal effects in two independent experiments (Figure 2B). Rucaparib did not significantly diminish *Mav*, *Stm* or MRSA CFUs, suggesting that the effect of rucaparib is specific for *Mtb*. These results imply that both rucaparib and niraparib inhibit specific species of bacteria through modeling of host signaling pathways.

Taken together, these data suggest that PARP activity is involved in *Mtb* survival in human macrophages, and that although there is some difference in their efficacy against *Mtb*-infected M1 compared to *Mtb*-infected M2, pharmacological inhibition of PARP is a promising strategy to target intracellular *Mtb*. Moreover, the effects of different PARP inhibitors are highly specific to certain pathogen species.

The Translational Potential of PARPi for Clinical Application

To evaluate the translational potential of PARPi for the development of HDT, we first tested whether PARPi displayed activity against macrophages infected with two different multi-drug resistant (MDR)-*Mtb* strains: an MDR-*Mtb* strain belonging to the Beijing genotype (strain 16319) and an MDR-*Mtb* Dutch outbreak strain (strain 2003-1128), both resistant to rifampicin (RIF) and isoniazid (INH). Although drug-susceptible and MDR *Mtb* strains are expected to respond similarly to HDT compounds, previous experiments by our laboratory suggested that in selective cases compound efficacy may differ between *Mtb* strains (20). Here, rucaparib significantly decreased intracellular MDR-*Mtb* and A-966492 showed activity against the MDR-*Mtb* Dutch outbreak strain (Figures 3A, B, n=6), highlighting the potential of PARPi to control intracellular outgrowth not only of drug-sensitive but also MDR-*Mtb* strains.

Next, the interaction of PARPi with first-line antibiotics was studied. Macrophages from five donors were infected with *Mtb* and treated with PARPi in the presence or absence of a non-toxic, suboptimal dose of RIF or INH (Figure 3C) (20, 21). As expected, PARPi significantly decreased *Mtb* CFUs in M1 (Figure 3D). In M2, however, several HDT compounds, including rucaparib, niraparib and positive control H-89 increased *Mtb* CFUs in two donors, illustrating the typically high variation in the response of donors to HDT compounds. Niraparib, pamiparib and rucaparib significantly enhanced the activity of INH against intracellular *Mtb* in M1. The coefficient of drug interaction (CID) of niraparib,

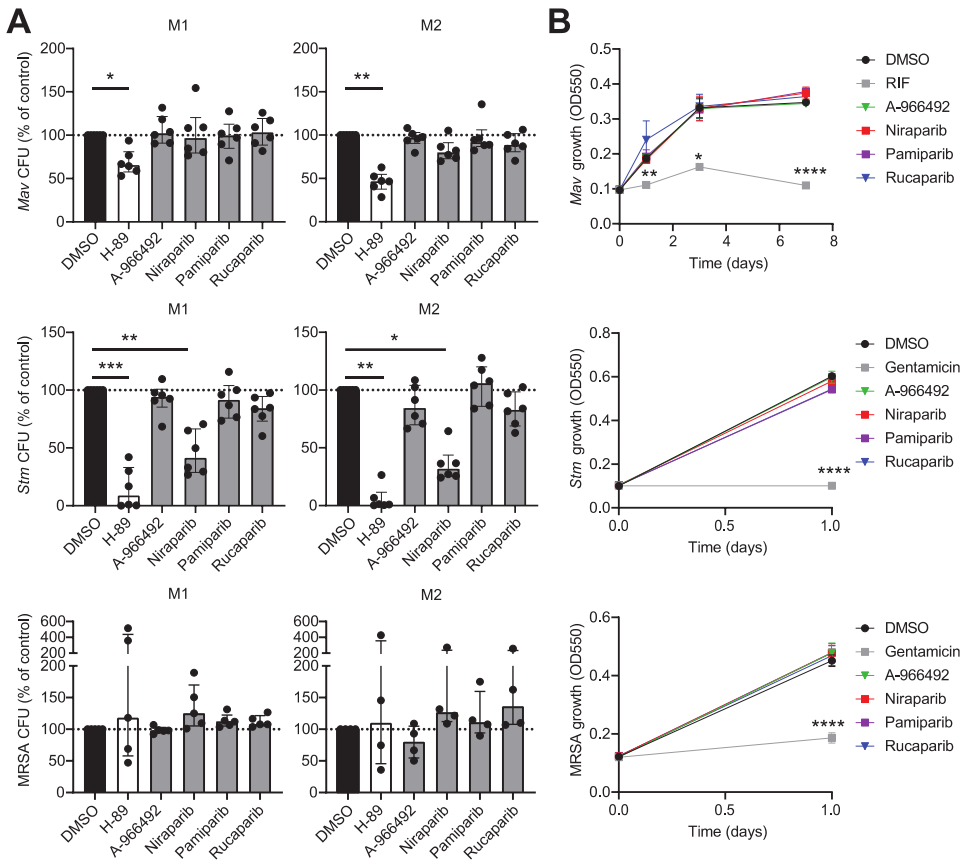


FIGURE 2 | PARPi niraparib decreases intracellular *Stm*. (A) *Mav*, *Stm* and MRSA CFUs after overnight treatment of infected M1 and M2 with PARPi (10 μ M), H-89 (10 μ M) or an equal volume of vehicle control DMSO (0.1% v/v). Data represent the median $\pm$ interquartile range from at least four different donors. Dots represent the mean from triplicate wells of a single donor. CFUs are expressed as percentage of vehicle control (i.e. DMSO). Differences were significant by Friedman's test with Dunn's multiple test correction against DMSO. (B) Liquid *Mav*, *Stm* and MRSA growth (in the absence of cells) was monitored during treatment with RIF (20 μ g/ml; positive control for *Mav*), gentamicin (50 μ g/ml; positive control for *Stm* and MRSA), PARPi (10 μ M) or vehicle control DMSO (0.1% v/v). Data represent the means $\pm$ S.D. of triplicate wells from a representative experiment out of two independent experiments. Differences were significant by RM two-way ANOVA with Dunnett's multiple test correction against DMSO. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

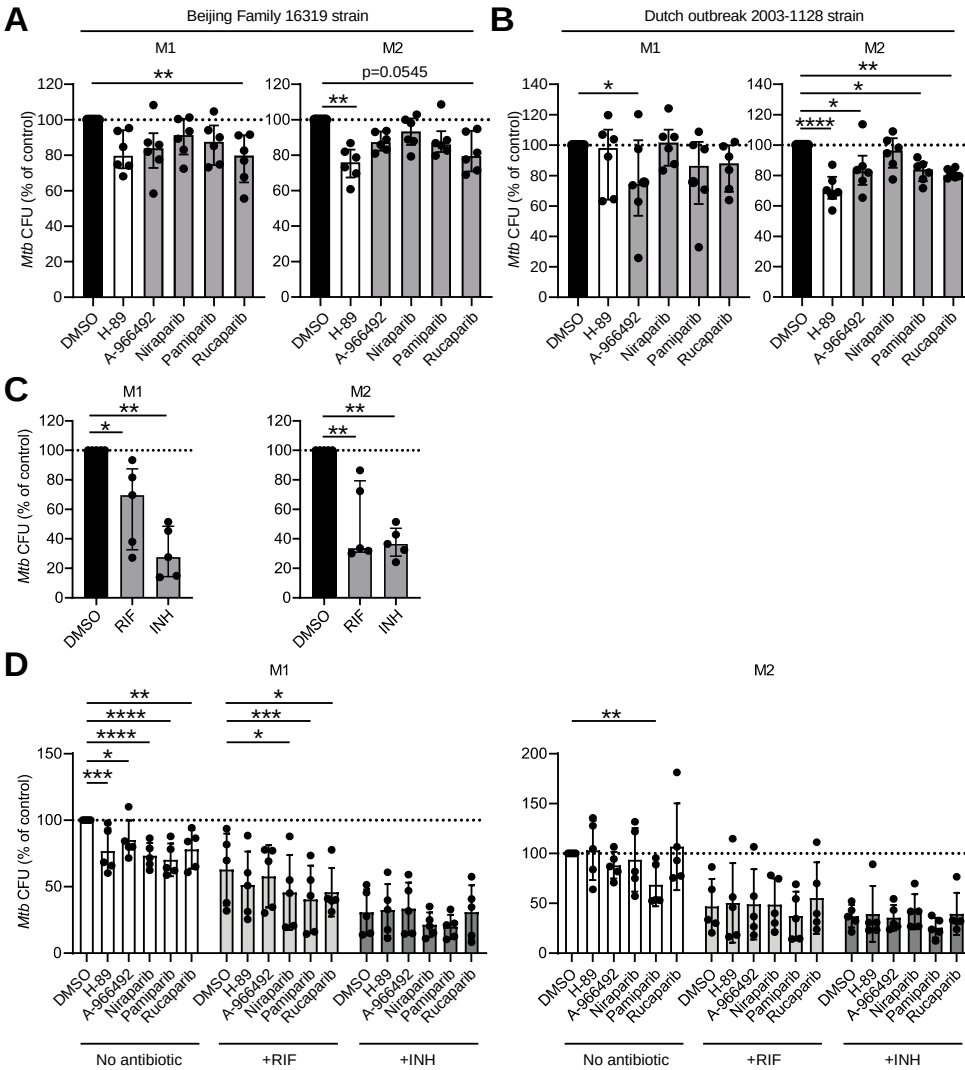


FIGURE 3 | The clinical potential of PARPi for host-directed therapy against *Mtb*. (A) Multidrug-resistant (MDR) *Mtb* CFUs after overnight treatment with PARPi (10 μ M), H-89 (10 μ M) or an equal volume of vehicle control DMSO (0.1% v/v) in M1 and M2. Data represent the median $\pm$ interquartile range from six different donors. Dots represent the mean from triplicate wells of a single donor. CFUs are expressed as percentage of control (i.e. DMSO). Differences were significant by Friedman's test with Dunn's multiple comparison test against DMSO. (B) MDR-*Mtb* CFUs after overnight treatment with PARPi (10 μ M), H-89 (10 μ M) or an equal volume of vehicle control DMSO (0.1% v/v) in M1 and M2. Differences were significant by RM one-way ANOVA with Dunnett's multiple comparison test against DMSO. (C) *Mtb* H37Rv CFUs after overnight treatment with RIF (0.05 μ g/ml), INH (0.4 μ g/ml) or an equal volume of vehicle control DMSO (0.1% v/v) in M1 and M2. Data represent the median $\pm$ interquartile range from five different donors. CFUs are expressed as percentage of control (i.e. DMSO). Differences were significant by RM one-way ANOVA with Dunnett's multiple

comparison test against DMSO. (D) *Mtb* H37Rv CFUs after overnight treatment with PARPi (10 μ M), H-89 (10 μ M) or an equal volume of vehicle control DMSO (0.1% v/v) in the presence or absence of a suboptimal dose of RIF (0.05 μ g/ml) or INH (0.4 μ g/ml) in M1 and M2. Data represent the median $\pm$ interquartile range from five different donors. CFUs are expressed as percentage of DMSO in the absence of antibiotics. Differences were significant by RM two-way ANOVA with Dunnett's multiple comparison test against the DMSO controls in the presence or absence of antibiotics. * p <0.05, ** p <0.01, *** p <0.001, **** p <0.001.

pamiparib and rucaparib with RIF was close to 1 in M1, suggesting that these PARPi had an additive effect to the antibiotic treatment and not a synergistic effect (Supplementary Table 1). Furthermore, compared with other PARPi, pamiparib seemed to enhance the activity of RIF in M2 and of INH in M1 and M2, albeit not significant.

PARPi Induce Immunomodulatory Effects in *Mtb*-Infected Human M1 and M2

To investigate whether the effect of PARPi against *Mtb* correlated with macrophage activation during *Mtb* infection, cytokine and chemokine production was quantified in the supernatants of *Mtb*-infected macrophages that were treated with PARPi or vehicle control DMSO overnight. *Mtb*-infection induced cytokine and chemokine production in M1 and M2 and tended to induce tumor necrosis factor (TNF)- α , macrophage inflammatory protein (MIP)-1 α , MIP-1 β and IP-10 and fractalkine in M1 and M2 (Figure 4A and Supplementary Table 2, n =8). Exposure to PARPi altered the cytokine and chemokine response of M1 and M2 upon *Mtb* infection (Figure 4B and Supplementary Table 2, n =4): pamiparib and rucaparib both increased MIP-1 α and MIP-1 β secretion by M1 and significantly decreased IFN- α 2 secretion by M2 compared to DMSO. Correlation plots between the cytokines and chemokines that were significantly modified by PARPi and *Mtb* CFUs did not identify a positive correlation between secretion profiles of macrophages and *Mtb* loads (Supplemental Figure 1). Collectively, these data suggest that pamiparib and rucaparib can modify the cytokine and chemokine response to *Mtb* infection.

Next, we investigated the expression levels of activation markers on the surface of *Mtb*-infected M1 and M2 that were treated with PARPi overnight. *Mtb*-infection tended to increase macrophage activation markers in M1 and M2 (Figure 4C, n =4). Surprisingly, rucaparib, but not other PARPi, tended to decrease expression of CD192 and CD209 and expression of human leucocyte antigen (HLA)-DR on the surface of *Mtb*-infected M1 and M2 (Figure 4D and Supplemental Figure 2, n =4). Furthermore, the expression of costimulatory molecule CD86 on the surface of M2 tended to be increased by rucaparib, and was significantly increased by pamiparib. These data suggest that rucaparib increases macrophage activation and possibly antigen presentation.

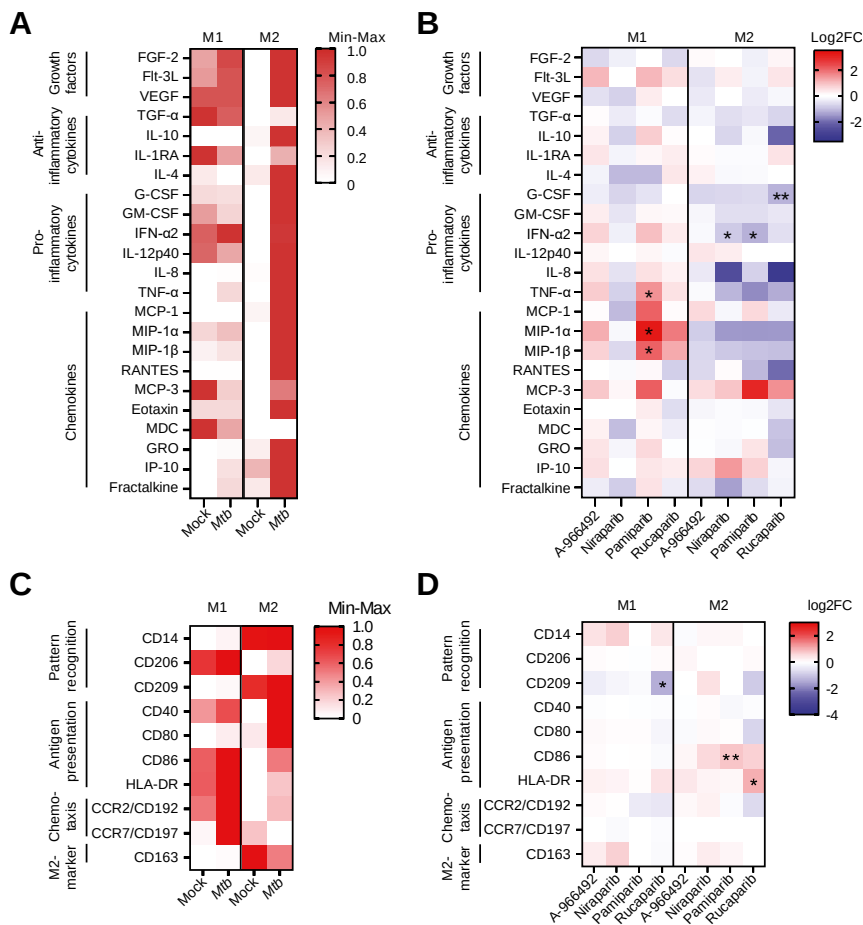


FIGURE 4 | PARP inhibitors induce immunomodulatory effects in human macrophages. (A) Heatmap displaying median cytokine/chemokine levels in the supernatants of *Mtb* H37Rv- or mock-infected M1 and M2 obtained from eight donors. Shown are the relative cytokine/chemokine secretion levels (>10 pg/ml) on a white to red color scale (min=0; max=1). Differences were tested using a Wilcoxon matched-pairs signed rank test. (B) Heatmap displaying median log₂ fold change (FC) cytokine/chemokine levels relative to vehicle control DMSO in the supernatants of *Mtb* H37Rv-infected M1 and M2 obtained from four donors. *Mtb* H37Rv-infected macrophages were exposed to PARPi (10 μ M) or an equal volume of DMSO (0.1% v/v) overnight. Differences were significant by Friedman's test with Dunn's multiple comparison test against DMSO. (C) Heatmap displaying relative geometric mean fluorescence intensity (gMFI) of proteins on the surface of Venus-expressing *Mtb* H37Rv-infected M1 and M2 obtained from four donors. Shown are the relative (geometric mean fluorescence intensity) gMFI levels on a white to red color scale (min=0; max=1). Differences were tested using a Wilcoxon matched-pairs signed rank test. (D) Heatmap displaying log₂ FC gMFI of proteins on the surface of Venus-expressing *Mtb* H37Rv-infected M1 and M2 obtained from four donors relative to vehicle control DMSO. Macrophages were exposed to PARPi (10 μ M) or an equal volume of DMSO (0.1% v/v) overnight. Differences were significant by Friedman's test with Dunn's multiple comparison test against DMSO. * $p < 0.05$, ** $p < 0.01$.

Given that several HDT compounds with demonstrated efficacy against intracellular *Mtb* in macrophages exert their activity by activating autophagy (22–25), we next assessed whether treatment with PARPi induced autophagy and lysosomal maturation. Accumulation of autophagic and lysosomal vesicles was quantified by confocal imaging on *Mtb*-infected and PARPi-treated macrophages from four donors that were stained with CYTO-ID or LysoTracker, respectively. Formation of autophagic or lysosomal vesicles could not be detected after 4h treatment with PARPi compared to DMSO (Supplemental Figures 3A, B). To examine the effect of PARPi on the autophagic flux, macrophages from three donors were treated with PARPi in the presence or absence of bafilomycin A1 (Baf) which is an autophagy inhibitor that prevents fusion between autophagosomes and lysosomes. As expected, treatment with Baf induced microtubule-associated protein light chain 3 (LC3)-II accumulation in M1 and M2 (Supplemental Figure 3C). However, PARPi did not increase LC3-II protein levels, regardless of the presence of Baf. Also, PARPi did not increase LAMP1 protein levels, suggesting that PARPi do not act via induction of autophagic and/or lysosomal pathways to control intracellular *Mtb* in human macrophages.

Collectively, our data demonstrate that PARPi modulate the immune response of *Mtb*-infected human macrophages via cytokine/chemokine expression and surface markers, which play a role in antigen presentation and chemotaxis. Our data also suggest that a role of induction of autophagy and phagosome maturation in PARP-induced *Mtb* control is not likely mechanistically involved in the mode of action.

Lactate Dehydrogenase, a Secondary Target of Rucaparib, Induces *Mtb* Control in Human Macrophages

Hexose-6-phosphate dehydrogenase (H6PD) and lactate dehydrogenase (LDH) were recently identified as additional target molecules of rucaparib (26). We therefore hypothesized that these metabolic targets might play a role in the more profound inhibitory effect of rucaparib on *Mtb* survival compared to other PARPi (Figures 1C, E, 3A, 5A). Lactate levels were significantly decreased in the supernatants of *Mtb*-infected M1 and M2 that had been treated with rucaparib compared to DMSO, suggesting that rucaparib indeed impaired LDH activity (Figure 5B, n=4). Interestingly, none of the other selected PARPi significantly affected lactate levels, compatible with the fact that none of these have been reported to modulate LDH activity. To study whether inhibition of H6PD or LDH could have contributed to the effect of rucaparib on *Mtb* control, we treated *Mtb*-infected macrophages with the specific LDH-inhibitor FX-11 or the G6PD-inhibitor 6-aminonicotinamide (6-AN) and determined intracellular *Mtb* levels in a flow cytometry-based assay, which allows a more rapid quantification of intracellular *Mtb* levels compared to classical CFU assays and generally showed a greater effect window of rucaparib

compared to CFU assay (Figure 1C versus 1E). Interestingly, FX-11 significantly decreased the percentage of *Mtb*-infected M1 (n=7) and M2 (n=8), whereas no effect of 6-AN on intracellular *Mtb* could be detected (Figure 5C). FX-11 did not affect the percentage of live M1 cells (n=6), but did decrease the percentage of live cells in a subset of M2 cultures (n=5). Together, these data suggest that inhibition of LDH, but not G6PD, likely contributed to the *Mtb*-decreasing effect of rucaparib. Corroborating this hypothesis, FX-11 treatment significantly decreased lactate levels in the supernatant of *Mtb*-infected M1, corresponding with decreased LDH activity (Figure 5E, n=6). FX-11 treatment did not result in decreased lactate levels in M2, which is likely a result of cellular toxicity in M2 (Figures 5D, E). Collectively, these data suggest that LDH could be involved in rucaparib-induced inhibition of *Mtb* and identifies LDH as potential host target during *Mtb* infections.

Discussion

In search of new potential treatments against TB, we have studied a series of PARPi, which are being considered for treatment of type 2 diabetes, for their ability to improve intracellular growth control by infected human M1 and M2 as novel candidate HDT therapeutics. Here, we showed that clinical compounds targeting PARP were able to decrease the intracellular survival of *Mtb*, suggesting that pharmacological inhibition of PARP can be a novel tool for TB treatment. PARPi had no direct antimycobacterial activity, suggesting that PARPi act in a HDT like fashion. In line with this, macrophage treatment with PARPi was associated with the modulation of macrophage function at the cytokine level and surface marker level. Lactate dehydrogenase (LDH) was identified as an additional target molecule for rucaparib's activity against intracellular *Mtb* which might explain the more profound inhibitory effect of rucaparib on *Mtb* survival compared to other PARPi. To our knowledge, the potential of PARPi as HDTs against *Mtb* has not been demonstrated before.

PARPs are host proteins involved in cellular processes acting via PARylation of their targets. It has recently been shown that PARP1 activation is triggered in mouse lungs upon *Mtb* infection (27). Importantly, genetic PARP1 depletion resulted in decreased lung bacterial burden in female mice. Here, we studied the role of PARP inhibition in a human and clinically relevant context. All clinical PARPi used in this study are known PARP1 and PARP2 inhibitors and have the ability to inhibit multiple proteins of the PARP family (28–31). Our data suggest that the efficacy of PARPi on bacterial clearance is specific for *Mtb* and does not translate to other intracellular bacteria, including the closely related species *Mav*. While not investigated here, this suggests that PARP is not a key target for MRSA, *Stm* and *Mav*, although the exact role of PARP during other bacterial infections remains to be resolved.

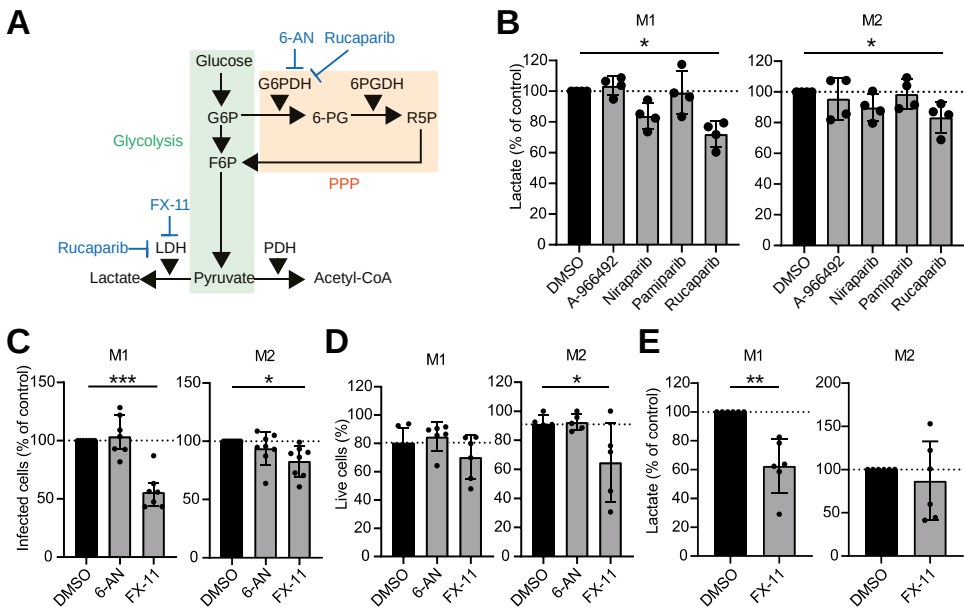


FIGURE 5 | Increased *Mtb* control by pharmacological inhibition of lactate dehydrogenase in human macrophages. (A) Schematic representation of metabolic pathway modulation by rucaparib, 6-AN and FX-11. Glycolysis is depicted in green; the pentose phosphate pathway (PPP) is depicted in orange. (B) Determination of lactate production in the supernatants of *Mtb* H37Rv-infected M1 and M2 that were treated with PARPi (10 μ M) or an equal volume of vehicle control DMSO (0.1% v/v) overnight. Data represent the median $\pm$ interquartile range from four different donors. Dots represent the mean from duplicate wells of a single donor. Differences were significant by RM one-way ANOVA with Dunnett's multiple comparison test against DMSO. (C) Percentage of M1 and M2 infected with Venus-expressing *Mtb* H37Rv that were treated with G6PD-inhibitor 6-aminonicotinamide (6-AN, 100 μ M) or LDH-inhibitor FX-11 (100 μ M) or an equal volume of vehicle control DMSO (0.1% v/v) overnight. Data represent the median $\pm$ interquartile range from at least seven different donors. Differences were significant by RM one-way ANOVA with Dunnett's multiple comparison test against DMSO. (D) Percentage of live M1 and M2 (i.e. PI-negative cells) after overnight treatment with 6-AN (100 μ M) or FX-11 (100 μ M) or an equal volume of vehicle control DMSO (0.1% v/v). Data represent the median $\pm$ interquartile range from at least five different donors. Differences were significant by RM two-way ANOVA with Dunnett's multiple test correction against DMSO. (E) Determination of lactate production in the supernatants of *Mtb* H37Rv-infected M1 and M2 that were treated with FX-11 (100 μ M) or an equal volume of vehicle control DMSO (0.1% v/v) overnight. Data represent the median $\pm$ interquartile range from six different donors. Differences were significant by RM one-way ANOVA with Dunnett's multiple comparison test against DMSO. *p < 0.05, **p < 0.01, *** p < 0.001.

Several PARP inhibitors, such as olaparib and talazoparib, can be metabolized by the hepatic enzyme CYP3A4, which is induced by the first-line TB antibiotic RIF (32–34). Rucaparib, however, is less sensitive to metabolism by CYP3A4 and the sensitivity of other PARPi remains to be determined. In our *in vitro* infection model, pamiparib had a significant additive effect to RIF in M1 and seemed to have an additive effect to RIF in M2 and to INH in both M1 and M2. This possible additive effect of pamiparib to RIF and INH in M2 was unexpected, since pamiparib did not show activity against *Mtb* in the absence of antibiotics in these cells. Although we observed differences in the cellular response to pamiparib in M1 versus M2 based on cytokine/chemokine secretion, it remains currently undefined which exact mechanisms were responsible for the difference in the efficacy of pamiparib against *Mtb* in M1 versus M2. This requires further investigation.

In addition to their role in DNA repair, mammalian PARPs display properties that are associated with and are likely to impact host-pathogen interactions. Firstly, PARPs are induced by the production of interferon-stimulated genes (ISGs), implicating a role during virus infection (35–38). The impact of PARP activity on virus replication remains controversial, as both promotion and reduction of viruses by PARP have both been described, which is likely dependent on the virus (as reviewed by 39). Moreover, an elevated type I IFN response correlates with progression to active TB in humans (40–42) and has been implicated in susceptibility to *Mtb* in mouse models (43, 44). Secondly, PARPs are involved in pro-inflammatory gene expression. Macrophages from PARP-deficient mice were unable to induce nuclear factor kappa B (NF- κ B)-mediated activation in response to lipopolysaccharide (LPS), limiting inducible nitric oxide synthase (iNOS), tumor necrosis factor alpha (TNF- α) and interferon gamma (IFN- γ) levels (45, 46). Here, we show that the inflammatory response of *Mtb*-infected macrophages is modified by pharmacological inhibition of PARP. At the cytokine level, pamiparib and rucaparib induced expression of chemokines MIP-1 α and MIP-1 β in M1. We furthermore show that the higher MIP-1 α and MIP-1 β levels did not correlate directly with increased inhibition of intracellular *Mtb*, compatible with their primary role in chemotactic cellular recruitment. Decreased MIP-1 α expression is associated with diabetes in *Mtb*-infected human monocyte-derived macrophages and in *Mtb*-infected diabetic mice (47–49). This suggests that macrophages obtained from diabetic patients or mice may be impaired in the recruitment of immune cells to *Mtb* sites of infection. Additionally, alveolar macrophages of diabetic mice feature decreased TNF- α secretion (47). Mice that have impaired TNF- α secretion and mice and humans treated with anti-TNF- α antibodies are more susceptible to *Mtb* infection, highlighting a direct protective effect of TNF- α against *Mtb* (50–52). Here, TNF- α was significantly induced by pamiparib treatment and it tended to be induced by A-966492 and rucaparib in M1. However, like for MIP-1 α , higher TNF- α levels did not

correlate directly with decreased intracellular *Mtb* levels. Together, these data demonstrate that PARPi treatment alters the cytokine/chemokine response of human macrophages during infection with *Mtb*. These effects are not directly correlated with intracellular *Mtb* control by macrophages, but these cytokines/chemokines could exert effects *in vivo* via other cells such as recruitment of immune cells. The potential of PARPi to improve recruitment of immune cells to sites of infection remains to be elucidated and could play an important role in the *in vivo* activity of PARPi against *Mtb*.

When evaluating the activation status of *Mtb*-infected macrophages in response to PARPi treatment, rucaparib appeared to increase the expression of HLA-DR and CD86, in contrast to other PARPi. As previously published, monocyte-derived macrophages (MDMs) obtained from type 2 diabetes (T2DM) patients displayed lower levels of HLA-DR and CD86 compared to MDMs obtained from healthy individuals upon *in vitro* *Mtb* infection (48). Collectively, these observations support the hypothesis that rucaparib is able to reverse diabetes-induced functional effects of macrophages and suggest that rucaparib could increase antigen presentation by human macrophages to specific T-cells.

Several studies have shown that diabetes was associated with a decrease in *Mtb* phagocytosis capacity in human and murine macrophages (47, 48, 53), possibly resulting from highly glycosylated proteins that impair recognition of bacterial components. Phagocytosis as mechanism of action of PARPi can be excluded in our current study, since PARPi were added well after phagocytosis, and further cell-to-cell exchange of *Mtb* bacteria was prevented by supplementing the culture medium with low dose gentamicin.

Multiple PARPi, including niraparib, rucaparib and talazoparib have recently been described to induce autophagy in cancer cell lines, as detected by increased LC3-I to LC3-II conversion on western blot analysis (54, 55). In our current study, induction of autophagy could not be detected, however, perhaps because non-replicating primary cells (M1, M2) were used instead of cell lines.

Proteome and metabolome profiling have recently revealed additional targets for PARPi which could contribute to *Mtb* control in our human macrophage infection model. *In vitro* kinase profiling showed that olaparib and rucaparib have a relatively similar affinity for members of the PARP family, but differ substantially in their affinity for protein kinases (29, 56). Our observation that rucaparib performed significantly better against *Mtb* than other PARPi suggests that unique kinases targeted by rucaparib might contribute to its effect against *Mtb*. The reported unique additional targets of rucaparib include cyclin dependent kinase 1 and 16 (CDK1, CDK16), pim-3 oncogene (PIM3), hexose-6-phosphate dehydrogenase (H6PD) and lactate dehydrogenase (LDH) (26, 28, 56, 57). Here we show that rucaparib decreased lactate production in *Mtb*-infected M1 and M2. Moreover, the specific LDH inhibitor FX-11 had a similar effect as rucaparib on intracellular *Mtb* load. This

suggests that LDH inhibition could be the major mechanism of action of rucaparib, and as such could be a promising target pathway in developing HDT against *Mtb*. In agreement with our findings, oral administration of FX-11 was recently described to decrease intracellular *Mtb* levels in mouse BMDM in a host-directed manner (58). Based on our data, we cannot exclude additional effects of rucaparib on further (secondary) targets during *Mtb* infection in human macrophages. Similarly, studying the potentially additive effects of the already described unique additional targets of niraparib are interesting in the development of novel HDT against *Mav* and *Stm*. Deoxynucleoside kinase (DCK) is a reported additional target of niraparib, and analysis of a published dataset of infected M2 showed that DCK is significantly upregulated 4h post *Stm*, but not *Mtb* infection (56, 59). Taken together, these data suggest that DCK is an interesting candidate for HDT against *Stm*.

There are several limitations of the current study. First, the current study has the limitation of small sample size. Although a small sample is common among studies with (primary) cells due to the laborious nature of these experiments, this could have resulted in insufficient statistical power between groups. Furthermore, we applied a single-dose administration of HDT compounds with a relatively short effect time (i.e. after overnight infection), which is an experimental setup that we generally use for the identification of novel HDT compounds (18, 20, 60). Although applying other compound concentrations, repeated doses or a longer treatment exposure could have resulted in a bigger effect size, the host targets (PARP and LDH) that we identified in our *in vitro* human macrophage model were recently published as host targets using *in vivo* mouse models as discussed above (27, 58). This agreeing set of independent findings underscores the power and validity of our approach, which aims to decipher novel pathways involved in host defense against intracellular bacterial infections in humans, and develop corresponding novel HDT.

In summary, we identified PARP as a host target for HDT during *Mtb* infections, and confirmed that PARP inhibition is a promising avenue in the development of HDT against *Mtb*. Furthermore, our data show that also off-target kinase pharmacology of PARP inhibitors may expand the current clinical scope of PARP inhibitors and that these molecules deserve serious consideration in the development of repurposed HDT against intracellular bacterial infections.

Materials and Methods

Reagents and Antibodies

Dimethyl sulfoxide (DMSO), staurosporine solution from *Streptomyces* sp. (STSP), H-89 dihydrochloride hydrate (H-89), bafilomycin A1 (Baf), 6-aminonicotinamide (6-AN), lactate dehydrogenase A inhibitor (FX-11), rifampicin (RIF), gentamicin, tetracycline

hydrochloride, ethylenediaminetetraacetic acid (EDTA) and Hoechst 33342 were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands). PARP inhibitors (A-966492, niraparib, pamiparib and rucaparib) and isoniazid (INH) were purchased from SelleckChem.

PE anti-human CD209 (1:50, clone 9E9A8), PerCP/Cy5.5 anti-human CD192 (CCR2, 1:20, clone K036C2), PE/Cy7 anti-human CD40 (1:100, clone 5C3), Alexa Fluor® 647 anti-human CD163 (1:50, clone CHI/61), APC/Cy7 anti-human CD206 (1:50, clone 15-2) and BV785 anti-human CD14 (1:200, clone M5E2) were purchased from Biolegend (London, United Kingdom). APC- R700 anti-human CD80 (1:200, clone L307.4), V500 anti-human HLA-DR (1:100, clone G46-6) and BV711 anti-human CD86 (1:100, clone 2331) were purchased from BD Bioscience (Vianen, The Netherlands). BV605 anti-human CD197 (CCR7, 1:200, clone G043H7) was purchased from Sony Biotechnology (Weybridge, United Kingdom). Mouse monoclonal anti-human beta actin (1:2000, clone mAbcam 8226) and rabbit polyclonal anti-human LAMP1 (1:500) were purchased from Abcam (Amsterdam, The Netherlands). Rabbit polyclonal anti-human LC3-I/II (1:250) was purchased from Cell Signaling Technology (Leiden, The Netherlands). Secondary stabilized peroxidase conjugated antibodies goat anti-rabbit IgG (H+L) (1:10,000) and goat anti-mouse IgG (H+L) (1:10,000) were purchased from Thermo Fisher Scientific (Breda, The Netherlands).

Cell Culture

Human peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats of healthy anonymous donors (Dutch, adults) after written informed consent (Sanquin Blood Bank, Amsterdam, The Netherlands) by density gradient centrifugation over Ficoll/amidotrizoat as described earlier (61). The use of buffy coats for research purposes was approved by the Institutional Review Board of the Leiden University Medical Center, The Netherlands. Magnetic cell sorting using anti-CD14-coated microbeads (Miltenyi Biotec, Auburn, CA) was used to isolate CD14⁺ monocytes. CD14⁺ cells were cultured for six days at 37°C/5% CO₂ in Gibco Roswell Park Memorial Institute (RPMI) 1640 medium (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS), 2 mM L-Alanyl-L-Glutamine (PAA, Linz, Austria), 100 units/ml penicillin (Thermo Fisher Scientific), 100 µg/ml streptomycin (Thermo Fisher Scientific) and either 5 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF, Thermo Fisher Scientific) to promote M1 differentiation or 50 ng/ml macrophage colony-stimulating factor (M-CSF, R&D Systems, Abingdon, United Kingdom) to promote M2 differentiation. Cytokines were added again at day 3 of differentiation in equal concentrations. The M1 and M2 macrophage phenotypes were subsequently validated by flow cytometry based surface marker expression (M1: CD14^{low}, CD163^{low}, CD11b^{high}; M2: CD14^{high}, CD163^{high}, CD11b^{low}) and by quantifying IL-10 and IL-12p40 production by Enzyme-Linked Immuno Sorbent Assay (ELISA) following stimulation of cells in the presence or absence of 100 ng/ml

lipopolysaccharide (LPS) for 24h (In vivoGen, San Diego, United States) as described before (60).

Bacterial Culture

Mycobacterial strains were cultured at 37°C in Difco™ Middlebrook 7H9 Broth (BD Bioscience) containing 10% acid- albumin-dextrose-catalase (ADC, BD Bioscience), 0.5% Tween- 80 (Sigma-Aldrich), 2% Glycerol (Sigma-Aldrich) and 50 µg/ml hygromycin B (Thermo Fisher Scientific) when appropriate. The following bacterial strains were used: *Mtb* H37Rv, Venus- expressing *Mtb* H37Rv (strain mc28120), DsRed-expressing *Mtb* H37Rv (18), MDR-*Mtb* Beijing family strain 16319 (62), MDR-*Mtb* Dutch outbreak strain 2003-1128 (62), *Mycobacterium avium* subsp. *avium* Chester strain 101 (ATCC, Wesel, Germany), DsRed-expressing *Salmonella enterica* serovar Typhimurium (*Stm*) strain SL1344 (18) and GFP-expressing methicillin-resistant *Staphylococcus aureus* (MRSA) strain USA300 JE2.

In Vitro Infection and Compound Treatment

Adherent cells were harvested by trypsinization and gentle scraping in FBS and seeded in RPMI 1640 medium supplemented with 10% FBS and 2 mM L-Alanyl-L-Glutamine on Costar 96-well flat bottom culture plates (30,000 cells/well) or on Costar 24-well flat bottom culture plates (300,000 cells/well) (Corning, Amsterdam, The Netherlands) and incubated overnight at 37°C/5% CO₂. Mycobacterial cultures were diluted to an early log-phase corresponding with an OD600 of 0.25 one day prior to infection in 7H9 broth containing 10% ADC, 0.5% Tween-80 and 2% Glycerol. DsRed-expressing *Stm* was recovered from frozen stock and cultured in Difco™ Luria-Bertani (LB) broth (BD Bioscience) containing 100 µg/ml ampicillin (Sigma-Aldrich) overnight at 37°C. Bacterial suspension was diluted 1:33 in LB broth and grown for 3- 4h to reach a log-phase with an OD600 between 0.4-0.6. GFP- expressing MRSA was recovered from frozen stock and cultured in Tryptic Soy (TS) broth (BD Bioscience) containing 5 µg/ml tetracycline hydrochlorine overnight at 37°C. Bacterial suspension was diluted 1:33 in TS broth and grown for 2-3h to reach a log- phase with an OD600 between 0.4-0.6. MRSA was harvested by centrifugation and resuspended in cold PBS supplemented with 5 mM EDTA. The indicated bacteria were diluted in RPMI 1640 medium (10% FBS and 2 mM L-Alanyl-L-Glutamine) to reach a multiplicity of infection (MOI) of 10. Accuracy of the MOI was validated by plating a serial dilution of the mycobacterial inoculum on Difco™ Middlebrook 7H10 agar (BD Bioscience) plates containing 10% oleic acid-albumin-dextrose-catalase (OADC, BD Bioscience) and 5% glycerol, by plating *Stm* on Difco™ LB agar plates (BD Bioscience) or by plating MRSA on TS agar (BD Bioscience). For mock infections, 7H9 broth was diluted in RPMI 1640 medium (10% FBS and 2 mM L-Alanyl-L-Glutamine) in equal concentrations (v/v) as the infection inoculum. Cells in flat-bottom 96-well plates

containing 100 μ l RPMI 1640 medium (10% FBS and 2 mM L-Alanyl-L-Glutamine) per well were inoculated with 100 μ l bacterial suspension or mock solution. Cells in flat-bottom 24-well plates containing 500 μ l RPMI 1640 medium (10% FBS and 2 mM L-Alanyl-L-Glutamine) per well were inoculated with 500 μ l bacterial suspension or mock solution. Plates were centrifuged for 3 min at 800 rpm to increase bacterial uptake and incubated for 1h for *Mtb*, *Mav* or MRSA infection or for 20 minutes for *Stm* infection at 37°C/5% CO₂. Extracellular bacteria were removed by washing with fresh RPMI 1640 medium supplemented with 10% FBS, 2 mM L-Alanyl-L-Glutamine and 30 μ g/ml gentamicin sulphate for 10 min. Cells were incubated at 37°C/5% CO₂ in RPMI 1640 medium supplemented with 10% FBS and 2 mM L-Alanyl-L-Glutamine and 5 μ g/ml gentamicin sulphate in the presence of H-89 (10 μ M), A-966492 (10 μ M), niraparib (10 μ M), pamiparib (10 μ M), rucaparib (10 μ M), 6-AN (100 μ M), FX-11 (100 μ M), Baf (10 nM), INH (0.4 mg/ml), RIF (0.05 mg/ml) or an equal amount of vehicle control DMSO (0.1% v/v) until readout. To calculate the coefficient of drug interaction (CID) the following formula was used: $CID = AB/(A \times B)$, where A indicates the ratio between antibiotic to the control group (DMSO, without antibiotics), B indicates the ratio between HDT compound to the control group and AB indicates the ratio between the treatments combined to the control group (63). A CID of 1 indicates an additive effect, <1 a synergistic effect and >1 an antagonistic effect.

CFU Assay

Cells in 96-well flat bottom plates (30,000 cells/well) were washed with PBS and lysed in 0.05% sodium dodecyl sulfate (SDS) solution (Thermo Fisher Scientific). Serially diluted cell lysates were plated on 7H10 Agar containing 10% OADC and 5% Glycerol (*Mtb* and *Mav*), LB agar (*Stm*) or TS agar (MRSA) and incubated at 37°C. CFUs were determined from triplicate wells.

Cellular Toxicity Assay

The number and percentage of dead cells based on plasma membrane integrity of the adherent cell population was quantified by analysis of microscopy images. Cells in 96-well flat bottom plates (30,000 cells/well) were stained with 2 μ g/ml propidium iodide (Sigma-Aldrich) and 2 μ g/ml Hoechst 33342 (H3570, Sigma-Aldrich) in 40 μ l/well phenol red-free RPMI (Sigma-Aldrich) supplemented with 10% FBS and 2 mM L-Alanyl-L-Glutamine and incubated for 5 min at room temperature (RT). Cells were imaged using a Leica AF6000 LC fluorescence microscope (Leica Microsystems, Wetzlar, Germany) combined with a 10x dry objective. Total and dead cell numbers were quantified by respectively counting the nuclei and the number of propidium iodide-positive cells using ImageJ software (64). STSP (2.5 μ M) was included as a positive control for cell death (Supplemental Figure 4).

Extracellular Bacterial Growth Assay

Compounds were diluted in Difco™ Middlebrook 7H9 Broth (*Mtb*, *Mav*), Difco™ LB Broth (*Stm*) or TS Broth (MRSA) and were added to log-phase bacterial cultures in flat bottom 96-well plates (OD₆₀₀ of 0.1). RIF (20 µg/ml) was added as positive control for reduction of *Mtb* and *Mav* growth and gentamicin (50 µg/ml) was added as positive control for reduction of *Stm* and MRSA growth. Bacterial plates were incubated at 37°C. Absorbance was measured directly after plating and at indicated times at a 550 nm wavelength on a Mithras LB 940 plate reader (Berthold Technologies, Bad Wildbad, Germany).

Western Immunoblot Analysis

Cells (300,000 cells/well in 24-wells plates) were lysed with 100 µl/ well EBSB buffer (10% v/v glycerol, 3% SDS, 100 mM Tris-HCl, pH 6.8) supplemented with one tablet of cOmplete™ EDTA-free protease inhibitor cocktail (Sigma-Aldrich) and one tablet of phosphatase inhibitor cocktail (PhosSTOP EASYpack, Sigma- Aldrich) per 10 ml. Cell lysates were boiled for 10 minutes at 95° C and stored at -20°C until use. Total protein concentrations were determined using a Pierce™ BCA protein assay kit (Thermo Fisher Scientific) according to manufacturer' instructions and protein concentrations were equalized and diluted in Laemmli sample buffer (Biorad) containing b-mercaptoethanol (Sigma-Aldrich). Samples were loaded on a 15-well 4–20% Mini-PROTEAN® TGX™ Precast Protein Gel (Bio-Rad Laboratories, Veenendaal, the Netherlands) and Amersham ECL Rainbow Molecular Weight Marker was added as reference (Sigma-Aldrich). Proteins were transferred to methanol-activated Immun-Blot PVDF membranes (Biorad) in Tris-glycine buffer (25 mM Tris, 192 mM glycine and 20% methanol). Membranes were blocked for 1h in polysorbate 20 tris-buffered saline (TTBS) supplemented with 5% w/v non-fat dry milk and incubated with the indicated antibodies in 5% non-fat dry milk/TTBS overnight at 4°C. Membranes were washed thrice for 15 min in TTBS and stained with secondary antibodies in 5% non-fat dry milk/TTBS for 2h at RT. Membranes were washed for 30 min with TTBS prior to revelation using enhanced chemiluminescence (ECL)™ Prime Western Blotting System reagent (GE Healthcare, Hoevelaken, The Netherlands). Imaging was performed on ChemiDoc XRS+ (Bio-Rad) or on an iBright Imaging System (Invitrogen, Breda, The Netherlands). Protein bands were quantified using ImageJ/Fiji software (64) and normalized to actin.

Cytokine and Chemokine Secretion by Multiplex Beads Assay

Human cytokine/chemokine levels were determined using the MilliPlex Human Cytokine/Chemokine magnetic bead premixed 41-plex kit (Millipore Billerica, MA, USA) as described before (60). Culture supernatants were collected and sterilized by using a 96-well filter plate containing a 0.2 µm PVDF membrane (Corning) following centrifugation. The following analytes were measured on a Bio-Plex 100 with Bio-Plex Manager™ software v6.1 (Biorad): sCD40L, EGF, FGF-2, Flt3 ligand, Fractalkine (CX3CL1), G-CSF, GM-

CSF, GRO (CXCL1), IFN- γ , IFN- α 2, IL-1 α , IL-1 β , IL-1RA, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8 (CXCL8), IL-9, IL-10, IL-12p40, IL-12p70, IL-13, IL-15, IL-17 α , IP-10 (CXCL10), MCP-1 (CCL2), MCP-3 (CCL7), MDC (CCL22), MIP-1 α (CCL3), MIP-1 β (CCL4), PDGF-AB/BB, RANTES (CCL5), TGF- α , TNF- α , TNF- β , VEGF, Eotaxin (CCL11) and PDGF-AA.

Flow Cytometry

Adherent and floating cells (300,000 cells/well in 24-wells plates) were washed in PBS/0.1% BSA (Merck, Darmstadt, Germany) and Fc receptors were blocked with 5% human serum (HS) in PBS for 10 min at RT. Cells were washed in PBS/0.1% BSA and stained with antibodies diluted in PBS/0.1% BSA for 30 min at 4°C. Cells were washed in PBS/0.1% BSA and fixed with 1% paraformaldehyde for 1h at 4°C. Fluorescence minus one (FMO) control samples were included to define background fluorescence for each stain.

Fluorescence staining was measured using a FACSLytic™ flow cytometer with FACSDiva software (BD Bioscience). Geometric mean fluorescence intensity (gMFIs) was recorded for each sample. Data were analyzed using FlowJo v10 software.

Lactate Assay

Cell culture supernatants were collected and sterilized by using a 96-well filter plate containing a 0.2 μ m PVDF membrane (Corning) following centrifugation. Undiluted sodium l-lactate (Sigma-aldrich) standard (0-8 mM, 5 μ l) or sample (5 μ l) was added to a flat-bottom 96-well plate (Greiner Bio-One, Alphen a/d Rijn, The Netherlands). 200 μ l of reaction mix (0.74 mM NAD, Roche Applied Science, Woerden, The Netherlands; 0.4 mM glycine, Sigma-Aldrich; 0.4 M hydrazine hydrate, Sigma-Aldrich) was added to allow conversion of lactate to pyruvate by lactate dehydrogenase (LDH): Lactate + NAD $^{+}$ $\leftrightarrow$ Pyruvate + NADH + H $^{+}$. Hydrazine hydrate was added to avoid conversion of the newly formed pyruvate back to lactate. Baseline NADH levels were measured using the SpectraMax i3x plate reader at OD340 before the addition of 2 μ l of three times diluted LDH from rabbit muscle (Roche Applied Science) to each well to initiate the lactate to pyruvate conversion. Following the addition of LDH, plates were incubated on a shaker at RT for 90 mins and the OD340 was measured again using a SpectraMax i3x plate reader.

Immunostaining and Confocal Microscopy

For confocal microscopy, cells were cultured on pre-washed black glass bottom poly-d-lysine coated 96-well plates (no. 1.5, MatTek Corporation, Ashland, MA, USA) at a density of 30,000 cells/well in RPMI 1640 medium supplemented with 10% FBS and 2 mM L-Alanyl-L-Glutamine and incubated overnight at 37° C/5% CO $_2$. Cells were infected with DsRed-expressing *Mtb* H37Rv as described above and treated with compound (10 μ M) or an equal volume of DMSO (0.1% v/v) for 4h. Culture medium was replaced with a solution of 75 nM LysoTracker® Deep Red (Thermo Fisher Scientific) and 1x CYTO-ID® Green (1:500, Enzo Life

Sciences, Bruxelles, Belgium) for 30 min at 37°C/5% CO₂ to stain lysosomal and autophagic vesicles, respectively. Cells were washed twice with PBS and fixed with Pierce™ 1% w/v formaldehyde (Thermo Fisher Scientific) for 1h at RT, washed twice with PBS and then stored at 4°C. Prior to imaging, plates were incubated with 2 µg/ml Hoechst 33342 for 5 min at RT and imaged using a SP8WLL confocal microscope (Leica) using a 63X oil immersion objective. Each treatment condition was performed in triplicate wells and three images were taken from each well. For image analysis, lysotracker and CYTO-ID channel background was subtracted using a rolling ball algorithm with a 10 pixel radius in Fiji/ImageJ (64). Lysotracker area and CYTO-ID area were specified for each image using Cellprofiler 3.1.9 (65) and were normalized to cell count based on Hoechst 33342 staining.

Statistical Analysis

All statistical analyses were carried out using GraphPad Prism 8 software (Graphpad Software, San Diego, CA, USA). Normal distribution of data sets was evaluated using the Shapiro-Wilk normality test. Correlation was evaluated with a Spearman's rank correlation test. Parametric paired data were tested with a paired t-test when comparing two groups and with RM one-way ANOVA or two-way ANOVA followed by Dunnett's multiple comparison test versus control when comparing three or more groups. Nonparametric paired data were tested with Wilcoxon matched-pairs signed rank test when comparing two groups and with Friedman's test followed by Dunn's multiple comparison test versus control when comparing three or more groups. Statistical tests were considered significant when $p < 0.05$ at 95% confidence interval.

Data Availability Statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics Statement

The studies involving human participants were reviewed and approved by Institutional Review Board of the Leiden University Medical Center, The Netherlands. The patients/participants provided their written informed consent to participate in this study.

Author Contributions

CD and TO designed the project and the main conceptual ideas. CD, SS, and KW performed the experiments and analyzed the experimental data. CD and TO wrote the manuscript. TO supervised the project. All authors contributed to the article and approved the submitted version.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

References

1. World Health Organization. *Global Tuberculosis Report*. Geneva: World Health Organization (2020). Licence: CC BY-NC-SA 3.0 IGO.
2. Muller J, Tanner R, Matsumiya M, Snowden MA, Landry B, Satti I, et al. Cytomegalovirus Infection Is a Risk Factor for TB Disease in Infants. *JCI Insight* (2019) 4(23):e130090. doi: 10.1172/jci.insight.130090
3. Jeon CY, Murray MB. Diabetes Mellitus Increases the Risk of Active Tuberculosis: A Systematic Review of 13 Observational Studies. *PLoS Med* (2008) 5(7):e152. doi: 10.1371/journal.pmed.0050152
4. Baker MA, Harries AD, Jeon CY, Hart JE, Kapur A, Lönnroth K, et al. The Impact of Diabetes on Tuberculosis Treatment Outcomes: A Systematic Review. *BMC Med* (2011) 9(1):81. doi: 10.1186/1741-7015-9-81
5. Lönnroth K, Roglic G, Harries AD. Improving Tuberculosis Prevention and Care Through Addressing the Global Diabetes Epidemic: From Evidence to Policy and Practice. *Lancet Diabetes Endocrinol* (2014) 2(9):730–9. doi: 10.1016/S2213-8587(14)70109-3
6. Huang Z, Luo Q, Guo Y, Chen J, Xiong G, Peng Y, et al. *Mycobacterium Tuberculosis*-Induced Polarization of Human Macrophage Orchestrates the Formation and Development of Tuberculous Granulomas *In Vitro*. *PLoS One* (2015) 10(6):e0129744–e. doi: 10.1371/journal.pone.0129744
7. Soriano F, Virág L, Jagtap P, SzabóÉ, Mabley J, Liaudet L, et al. Diabetic Endothelial Dysfunction: The Role of Poly(ADP-Ribose) Polymerase Activation. *Nat Med* (2001) 7:108–13. doi: 10.1038/83241
8. Pacher P, Liaudet L, Soriano FG, Mabley JG, Szabo E, Szabo C. The Role of Poly(ADP-Ribose) Polymerase Activation in the Development of Myocardial and Endothelial Dysfunction in Diabetes. *Diabetes* (2002) 51(2):514–21. doi: 10.2337/diabetes.51.2.514
9. Szabo C, Zanchi A, Komjati K, Pacher P, Krolewski AS, Quist WC, et al. Poly (ADP-Ribose) Polymerase Is Activated in Subjects at Risk of Developing Type 2 Diabetes and Is Associated With Impaired Vascular Reactivity. *Circulation* (2002) 106(21):2680–6. doi: 10.1161/01.CIR.0000038365.78031.9C
10. Sachdev E, Tabatabai R, Roy V, Rimel B, Mita MM. PARP Inhibition in Cancer: An Update on Clinical Development. *J Targeted Oncol* (2019) 14 (6):657–79. doi: 10.1007/s11523-019-00680-2
11. Burkart V, Wang Z-Q, Radons J, Heller B, Herceg Z, Stingl L, et al. Mice Lacking the Poly (ADP-Ribose) Polymerase Gene Are Resistant to Beta-Cell Destruction and Diabetes Development Induced by Streptozocin. *Nat Med* (1999) 5:314–9. doi: 10.1038/6535
12. Pieper AA, Brat DJ, Krug DK, Watkins CC, Gupta A, Blackshaw S, et al. Poly (ADP-Ribose) Polymerase-Deficient Mice Are Protected From Streptozotocin- Induced Diabetes. *Proc Natl Acad Sci USA* (1999) 96(6):3059–64. doi: 10.1073/ pnas.96.6.3059
13. Soriano FG, Pacher P, Mabley J, Liaudet L, Szabo C. Rapid Reversal of the Diabetic Endothelial Dysfunction by Pharmacological Inhibition of Poly (ADP-Ribose) Polymerase. *Circ Res* (2001) 89(8):684–91. doi: 10.1161/ hh2001.097797
14. Krug S, Parveen S, Bishai WR. Host-Directed Therapies: Modulating Inflammation to Treat Tuberculosis. *Front Immunol* (2021) 12:1247. doi: 10.3389/fimmu.2021.660916
15. Nile DL, Rae C, Hyndman IJ, Gaze MN, Mairs RJ. An Evaluation *In Vitro* of PARP-1 Inhibitors, Rucaparib and Olaparib, as Radiosensitisers for the Treatment of Neuroblastoma. *BMC Cancer* (2016) 16:621. doi: 10.1186/ s12885-016-2656-8
16. Cho H-y, Kim Y-B, Park W-h, No JH. Enhanced Efficacy of Combined Therapy With Checkpoint Kinase 1 Inhibitor and Rucaparib *via* Regulation of Rad51 Expression in BRCA Wild-Type Epithelial Ovarian Cancer Cells. *J Cancer Res Treat* (2021) 53(3):819–28. doi: 10.4143/crt.2020.1013

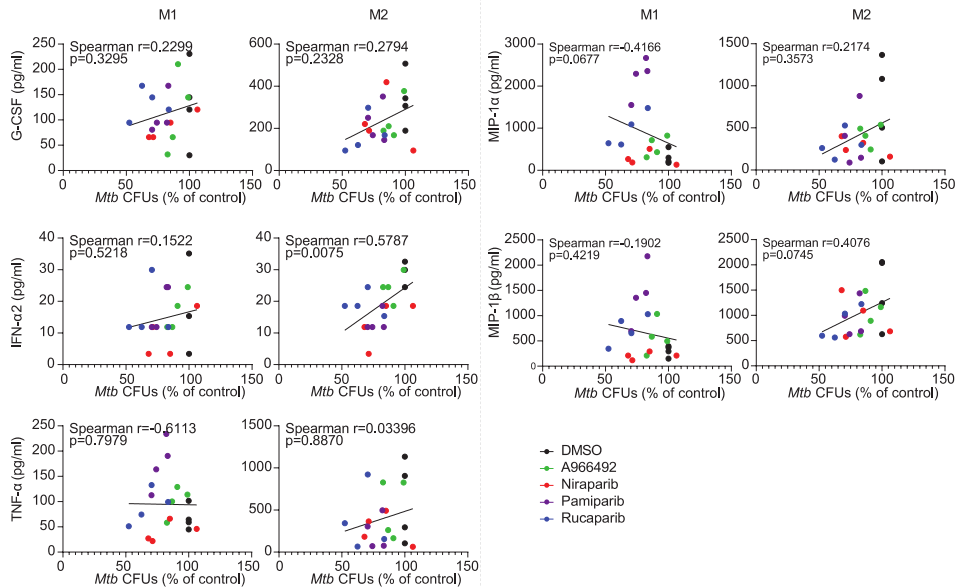
17. Smith HL, Prendergast L, Curtin NJ. Exploring the Synergy Between PARP and CHK1 Inhibition in Matched BRCA2 Mutant and Corrected Cells. *J Cancers* (2020) 12(4):878. doi: 10.3390/cancers12040878
18. Korbbee CJ, Heemskerk MT, Kocev D, van Strijen E, Rabiee O, Franken KLMC, et al. Combined Chemical Genetics and Data-Driven Bioinformatics Approach Identifies Receptor Tyrosine Kinase Inhibitors as Host-Directed Antimicrobials. *Nat Commun* (2018) 9(1):358. doi: 10.1038/s41467-017-02777-6
19. Kuijl C, Savage NDL, Marsman M, Tuin AW, Janssen L, Egan DA, et al. Intracellular Bacterial Growth Is Controlled by a Kinase Network Around PKB/Akt1. *Nature* (2007) 450(7170):725–30. doi: 10.1038/nature06345
20. Heemskerk MT, Korbbee CJ, Esselink J, dos Santos CC, van Veen S, Gordijn IF, et al. Repurposing Diphenylbutylpiperidine-Class Antipsychotic Drugs for Host-Directed Therapy of *Mycobacterium Tuberculosis* and Salmonella Enterica Infections. *bioRxiv* (2021) 11(1):19634. doi: 10.1038/s41598-021-98980-z
21. Singh M, Sasi P, Rai G, Gupta VH, Amarapurkar D, Wangikar PP. Studies on Toxicity of Antitubercular Drugs Namely Isoniazid, Rifampicin, and Pyrazinamide in an *In Vitro* Model of HepG2 Cell Line. *J Medicinal Chem Res* (2011) 20(9):1611–5. doi: 10.1007/s00044-010-9405-3
22. Rekha RS, Rao Muvva SSVJ, Wan M, Raqib R, Bergman P, Brighenti S, et al. Phenylbutyrate Induces LL-37-Dependent Autophagy and Intracellular Killing of *Mycobacterium Tuberculosis* in Human Macrophages. *Autophagy* (2015) 11(9):1688–99. doi: 10.1080/15548627.2015.1075110
23. Gutierrez MG, Master SS, Singh SB, Taylor GA, Colombo MI, Deretic V. Autophagy Is a Defense Mechanism Inhibiting BCG and *Mycobacterium Tuberculosis* Survival in Infected Macrophages. *Cell* (2004) 119(6):753–66. doi: 10.1016/j.cell.2004.11.038
24. Parihar SP, Guler R, Khutlang R, Lang DM, Hurdal R, Mhlanga MM, et al. Statin Therapy Reduces the *Mycobacterium Tuberculosis* Burden in Human Macrophages and in Mice by Enhancing Autophagy and Phagosome Maturation. *J Infect Dis* (2013) 209(5):754–63. doi: 10.1093/infdis/jit550
25. Singhal A, Jie L, Kumar P, Hong GS, Leow MK-S, Paleja B, et al. Metformin as Adjunct Antituberculosis Therapy. *Sci Trans Med* (2014) 6(263):263ra159–263ra159. doi: 10.1126/scitranslmed.3009885
26. Nonomiya Y, Noguchi K, Katayama K, Sugimoto Y. Novel Pharmacological Effects of Poly (ADP-Ribose) Polymerase Inhibitor Rucaparib on the Lactate Dehydrogenase Pathway. *Biochem Biophys Res Commun* (2019) 510(4):501–7. doi: 10.1016/j.bbrc.2019.01.133
27. Krug S, Ordóñez AA, Klunk M, Kang BG, Jain SK, Dawson TM, et al. Host Regulator PARP1 Contributes to Sex Differences and Immune Responses in a Mouse Model of Tuberculosis. *bioRxiv* (2021) 440820. doi: 10.1101/2021.04.21.440820
28. Antolin AA, Ameratunga M, Banerji U, Clarke PA, Workman P, Al-Lazikani B. The Kinase Polypharmacology Landscape of Clinical PARP Inhibitors. *Sci Rep* (2020) 10(1):2585. doi: 10.1038/s41598-020-59074-4
29. Carney B, Kossatz S, Lok BH, Schneeberger V, Gangangari KK, Pillarsetty NVK, et al. Target Engagement Imaging of PARP Inhibitors in Small-Cell Lung Cancer. *Nat Commun* (2018) 9(1):176. doi: 10.1038/s41467-017-02096-w
30. McGonigle S, Chen Z, Wu J, Chang P, Kolber-Simonds D, Ackermann K, et al. E7449: A Dual Inhibitor of PARP1/2 and Tankyrase1/2 Inhibits Growth of DNA Repair Deficient Tumors and Antagonizes Wnt Signaling. *Oncotarget* (2015) 6(38):41307–23. doi: 10.18632/oncotarget.5846
31. Thorsell A-G, Schüler H. Selectivity Profile of the Poly(ADP-Ribose) Polymerase (PARP) Inhibitor, A-966492. *bioRxiv* (2017) 119818. doi: 10.1101/119818

32. Ringley JT, Moore DC, Patel J, Rose MS. Poly (ADP-Ribose) Polymerase Inhibitors in the Management of Ovarian Cancer: A Drug Class Review. *P T* (2018) 43(9):549–56.
33. Glaeser H, Drescher S, Eichelbaum M, Fromm MF. Influence of Rifampicin on the Expression and Function of Human Intestinal Cytochrome P450 Enzymes. *Br J Clin Pharmacol* (2005) 59(2):199–206. doi: 10.1111/j.1365- 2125.2004.02265.x
34. Attwa MW, Kadi AA, Abdelhameed AS, Alhazmi HA. Metabolic Stability Assessment of New PARP Inhibitor Talazoparib Using Validated LC-MS/MS Methodology: *In Silico* Metabolic Vulnerability and Toxicity Studies. *Drug Des Devel Ther* (2020) 14:783–93. doi: 10.2147/DDDT.S239458
35. Liu S-Y, Sanchez DJ, Aliyari R, Lu S, Cheng G. Systematic Identification of Type I and Type II Interferon-Induced Antiviral Factors. *Proc Natl Acad Sci* (2012) 109(11):4239. doi: 10.1073/pnas.1114981109
36. Atasheva S, Akhrymuk M, Frolova EI, Frolov I. New PARP Gene With an Anti-Alphavirus Function. *J Virol* (2012) 86(15):8147–60. doi: 10.1128/JVI.00733-12
37. Caprara G, Prosperini E, Piccolo V, Sigismondo G, Melacarne A, Cuomo A, et al. PARP14 Controls the Nuclear Accumulation of a Subset of Type I IFN- Inducible Proteins. *J Immunol* (2018) 200(7):2439. doi: 10.4049/jimmunol.1701117
38. Zhang Y, Mao D, Roswit WT, Jin X, Patel AC, Patel DA, et al. PARP9-DTX3L Ubiquitin Ligase Targets Host Histone H2BJ and Viral 3C Protease to Enhance Interferon Signaling and Control Viral Infection. *Nat Immunol* (2015) 16(12):1215–27. doi: 10.1038/ni.3279
39. Fehr AR, Singh SA, Kerr CM, Mukai S, Higashi H, Aikawa M. The Impact of PARPs and ADP-Ribosylation on Inflammation and Host-Pathogen Interactions. *Genes Dev* (2020) 34(5-6):341–59. doi: 10.1101/gad.334425.119
40. Zak DE, Penn-Nicholson A, Scriba TJ, Thompson E, Suliman S, Amon LM, et al. A Blood RNA Signature for Tuberculosis Disease Risk: A Prospective Cohort Study. *Lancet* (2016) 387(10035):2312–22. doi: 10.1016/S0140-6736 (15)01316-1
41. Scriba TJ, Penn-Nicholson A, Shankar S, Hraha T, Thompson EG, Sterling D, et al. Sequential Inflammatory Processes Define Human Progression From M. Tuberculosis Infection to Tuberculosis Disease. *PLoS Pathog* (2017) 13(11): e1006687. doi: 10.1371/journal.ppat.1006687
42. Ottenhoff TH, Dass RH, Yang N, Zhang MM, Wong HE, Sahiratmadja E, et al. Genome-Wide Expression Profiling Identifies Type 1 Interferon Response Pathways in Active Tuberculosis. *PLoS One* (2012) 7(9):e45839. doi: 10.1371/ journal.pone.0045839
43. Ji DX, Yamashiro LH, Chen KJ, Mukaida N, Kramnik I, Darwin KH, et al. Type I Interferon-Driven Susceptibility to *Mycobacterium Tuberculosis* Is Mediated by IL-1ra. *Nat Microbiol* (2019) 4(12):2128–35. doi: 10.1038/s41564-019-0578-3
44. Dorhoi A, Yeremeev V, Nouailles G, Weiner J3rd, Jörg S, Heinemann E, et al. Type I IFN Signaling Triggers Immunopathology in Tuberculosis-Susceptible Mice by Modulating Lung Phagocyte Dynamics. *Eur J Immunol* (2014) 44(8):2380–93. doi: 10.1002/eji.201344219
45. Oliver FJ, Ménissier-de Murcia J, Nacci C, Decker P, Andriantsitohaina R, Muller S, et al. Resistance to Endotoxin Shock as a Consequence of Defective NF-kappaB Activation in Poly (ADP-Ribose) Polymerase-1 Deficient Mice. *EMBO J* (1999) 18(16):4446–54. doi: 10.1093/emboj/18.16.4446
46. Hassa PO, Hottiger MO. A Role of Poly (ADP-Ribose) Polymerase in NF- kappaB Transcriptional Activation. *Biol Chem* (1999) 380(7-8):953–9. doi: 10.1515/BC.1999.118
47. Alim MA, Kupz A, Sikder S, Rush C, Govan B, Ketheesan N. Increased Susceptibility to *Mycobacterium Tuberculosis* Infection in a Diet-Induced Murine Model of Type 2 Diabetes. *Microbes infection* (2020) 22(8):303–11. doi: 10.1016/j.micinf.2020.03.004
48. Lopez-Lopez N, Martinez AGR, Garcia-Hernandez MH, Hernandez-Pando R, Castaneda-Delgado JE, Lugo-Villarino G, et al. Type-2 Diabetes Alters the Basal Phenotype of Human Macrophages and Diminishes Their Capacity to Respond, Internalise, and Control *Mycobacterium Tuberculosis*. *Memorias do Instituto Oswaldo Cruz* (2018) 113(4):e170326. doi: 10.1590/0074-

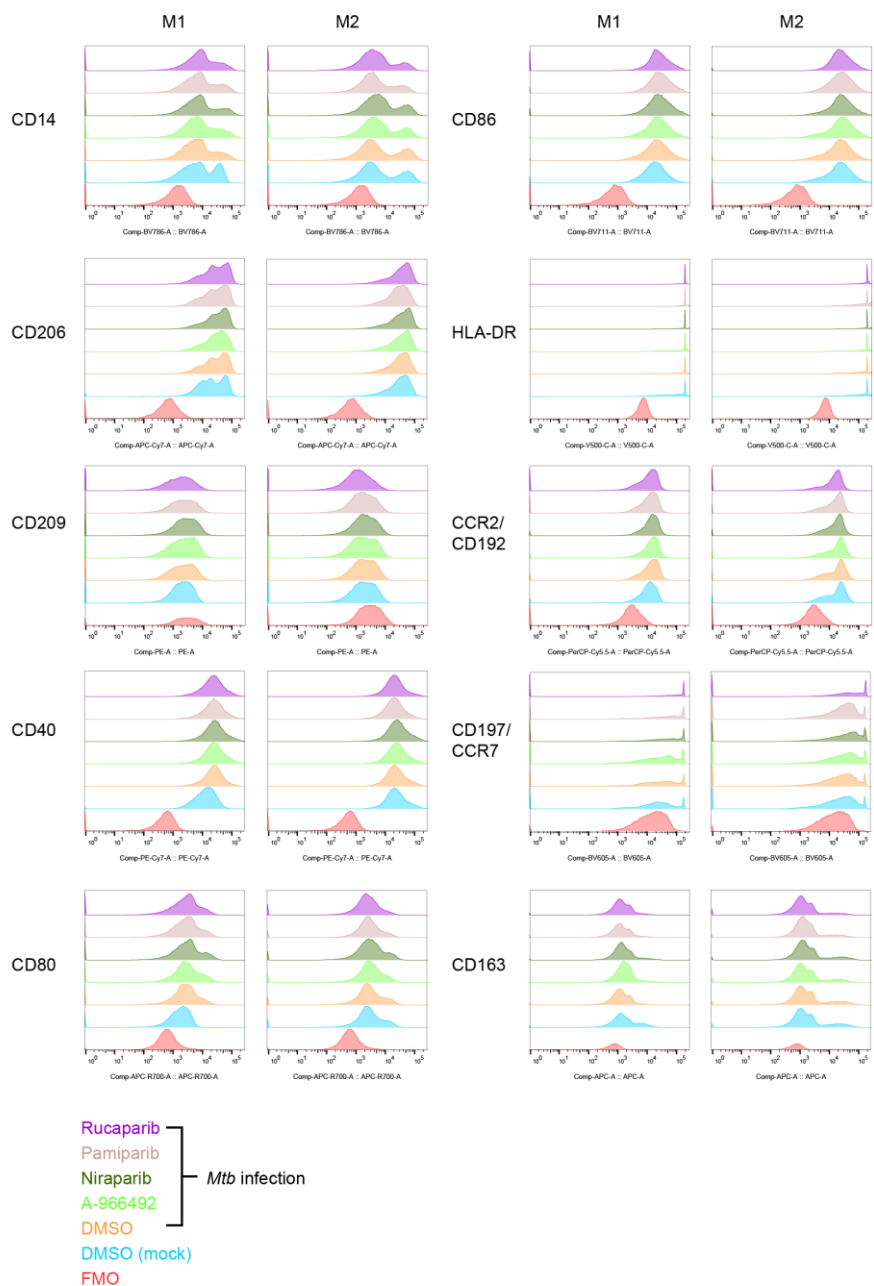
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49. Vallerskog T, Martens GW, Kornfeld H. Diabetic Mice Display a Delayed Adaptive Immune Response to *Mycobacterium Tuberculosis*. *J Immunol* (2010) 184(11):6275. doi: 10.4049/jimmunol.1000304
 50. Flynn JL, Goldstein MM, Chan J, Triebold KJ, Pfeffer K, Lowenstein CJ, et al. Tumor Necrosis Factor-Alpha Is Required in the Protective Immune Response Against *Mycobacterium Tuberculosis* in Mice. *Immunity* (1995) 2 (6):561–72. doi: 10.1016/1074-7613(95)90001-2
 51. Keane J, Gershon S, Wise RP, Mirabile-Levens E, Kasznica J, Schwieterman WD, et al. Tuberculosis Associated With Infliximab, a Tumor Necrosis Factor Alpha-Neutralizing Agent. *N Engl J Med* (2001) 345(15):1098–104. doi: 10.1056/NEJMoa011110
 52. Mohan VP, Scanga CA, Yu K, Scott HM, Tanaka KE, Tsang E, et al. Effects of Tumor Necrosis Factor Alpha on Host Immune Response in Chronic Persistent Tuberculosis: Possible Role for Limiting Pathology. *Infection Immun* (2001) 69(3):1847. doi: 10.1128/IAI.69.3.1847-1855.2001
 53. Martinez N, Ketheesan N, West K, Vallerskog T, Kornfeld H. Impaired Recognition of *Mycobacterium Tuberculosis* by Alveolar Macrophages From Diabetic Mice. *J Infect Dis* (2016) 214(11):1629–37. doi: 10.1093/infdis/jiw436
 54. Santiago-O’Farrill JM, Weroha SJ, Hou X, Pang L, Rask P, Lu Z, et al. PARP Inhibitor-Induced Autophagy Provides an Adaptive Mechanism of Drug Resistance in Preclinical Models of Ovarian Cancer. *AACR* (2019) 126 (4):894–907. doi: 10.1158/1538-7445.AM2019-4768
 55. Liu Y, Song H, Song H, Feng X, Zhou C, Huo Z. Targeting Autophagy Potentiates the Anti-Tumor Effect of PARP Inhibitor in Pediatric Chronic Myeloid Leukemia. *AMB Express* (2019) 9(1):108. doi: 10.1186/s13568-019- 0836-z
 56. Antolín AA, Mestres J. Linking Off-Target Kinase Pharmacology to the Differential Cellular Effects Observed Among PARP Inhibitors. *Oncotarget* (2014) 5(10):3023–8. doi: 10.18632/oncotarget.1814
 57. Knezevic CE, Wright G, Rix LLR, Kim W, Kuenzi BM, Luo Y, et al. Proteome- Wide Profiling of Clinical PARP Inhibitors Reveals Compound-Specific Secondary Targets. *Cell Chem Biol* (2016) 23(12):1490–503. doi: 10.1016/j.chembiol.2016.10.011
 58. Krishnamoorthy G, Kaiser P, Abed UA, Weiner J3rd, Moura-Alves P, Brinkmann V, et al. FX11 Limits *Mycobacterium Tuberculosis* Growth and Potentiates Bactericidal Activity of Isoniazid Through Host-Directed Activity. *Dis Models Mech* (2020) 13(3):dmm041954. doi: 10.1242/dmm.041954
 59. Blischak JD, Tailleux L, Mitrano A, Barreiro LB, Gilad Y. Mycobacterial Infection Induces a Specific Human Innate Immune Response. *Sci Rep* (2015) 5(1):16882. doi: 10.1038/srep16882
 60. Moreira JD, Koch BE, van Veen S, Walburg KV, Vrieling F, Mara Pinto Dabés Guimarães T, et al. Functional Inhibition of Host Histone Deacetylases (HDACs) Enhances *In Vitro* and *In Vivo* Anti-Mycobacterial Activity in Human Macrophages and in Zebrafish. *Front Immunol* (2020) 11:36. doi: 10.3389/fimmu.2020.00036
 61. Verreck FA, de Boer T, Langenberg DM, Hoeve MA, Kramer M, Vaisberg E, et al. Human IL-23-Producing Type 1 Macrophages Promote But IL-10- Producing Type 2 Macrophages Subvert Immunity to (Myco)Bacteria. *Proc Natl Acad Sci USA* (2004) 101(13):4560–5. doi: 10.1073/pnas.0400983101
 62. Kremer K, van Soolingen D, Frothingham R, Haas WH, Hermans PWM, Martín C, et al. Comparison of Methods Based on Different Molecular Epidemiological Markers for Typing of *Mycobacterium Tuberculosis* Complex Strains: Interlaboratory Study of Discriminatory Power and Reproducibility. *J Clin Microbiol* (1999) 37(8):2607–18. doi: 10.1128/JCM.37.8.2607-2618.1999
 63. Cao S-s, Zhen Y-s. Potentiation of Antimetabolite Antitumor Activity *In Vivo* by Dipyridamole and Amphotericin B. *Cancer Chemotherapy Pharmacol* (1989) 24(3):181–6. doi: 10.1007/BF00300240

64. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, et al. Fiji: An Open-Source Platform for Biological-Image Analysis. *Nat Methods* (2012) 9(7):676–82. doi: 10.1038/nmeth.2019
65. Carpenter AE, Jones TR, Lamprecht MR, Clarke C, Kang IH, Friman O, et al. CellProfiler: Image Analysis Software for Identifying and Quantifying Cell Phenotypes. *Genome Biol* (2006) 7(10):R100. doi:10.1186/gb-2006-7-10-r100

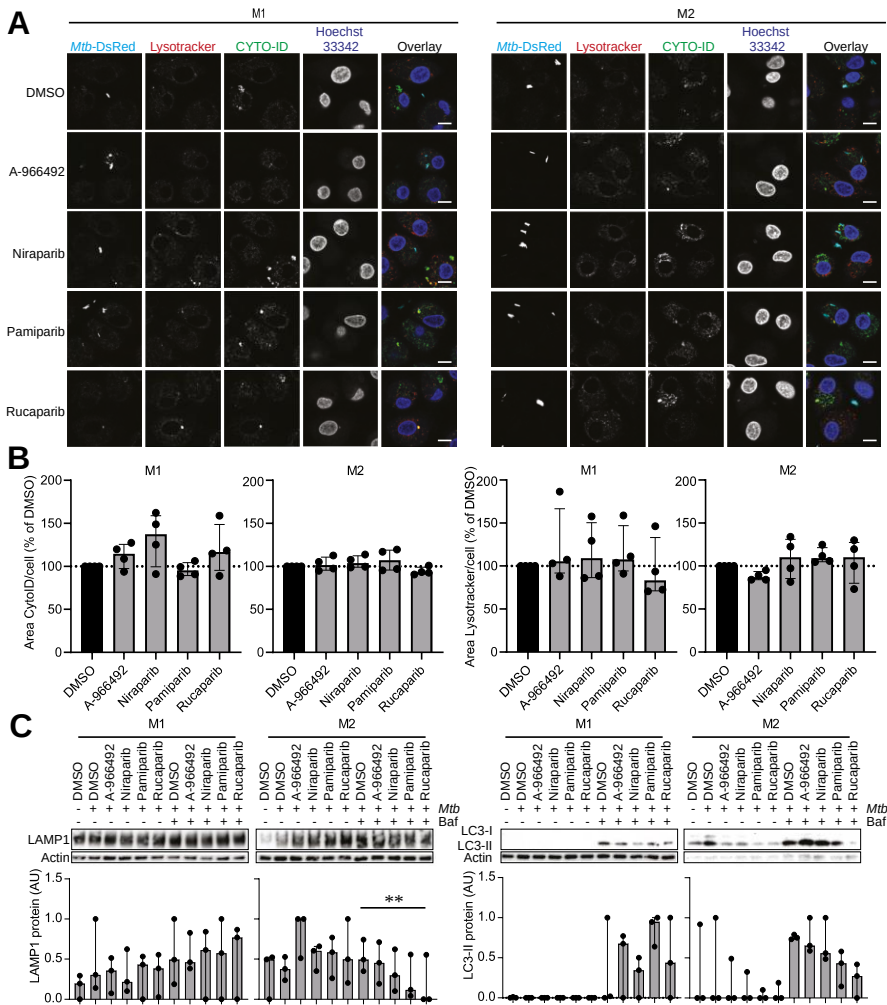
Supplementary Figures



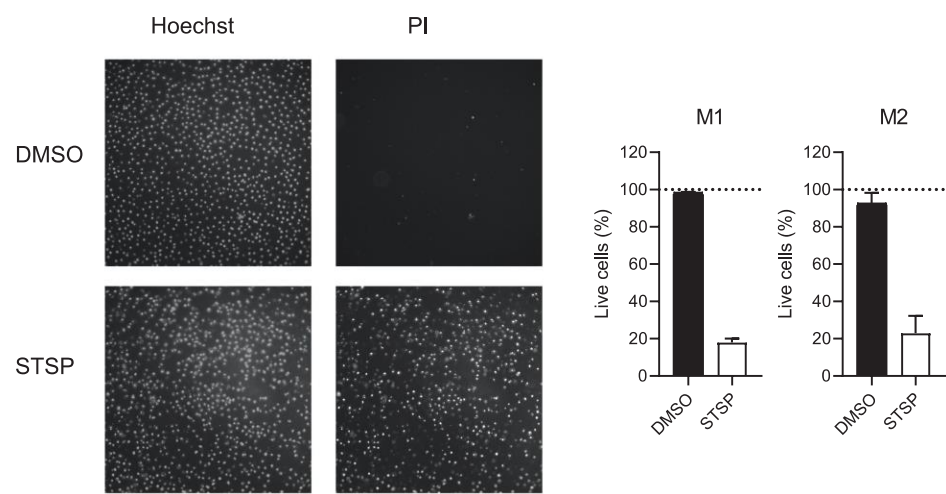
SUPPLEMENTAL FIGURE 1 | Correlation plots do not identify a positive correlation between chemokine or cytokine production and *Mtb* control. Correlation between *Mtb* H37Rv outgrowth and cytokine/chemokine secretion levels in the supernatants of M1 (left) and M2 (right) cultures. *Mtb* H37Rv CFU counts are expressed as a percentage of control (i.e. DMSO). Every symbol represents data from one donor with a total of four donors. A linear regression was drawn through the data points and the correlation was determined using a Spearman's rank correlation coefficient.



SUPPLEMENTAL FIGURE 2 | Exposure to PARPi alters surface marker expression on M1 and M2 upon *Mtb* infection. Histograms showing fluorescence intensity of M1 and M2 macrophages that were either mock infected or infected with Venus-expressing *Mtb* H37Rv and treated with PARPi (10 μ M) or an equal volume of vehicle control DMSO (0.1% v/v) overnight. Concatenated samples obtained from four donors are shown.



SUPPLEMENTAL FIGURE 3 | A role of autophagy or lysosomal maturation in the PARPi-induced protection against *Mtb* could not be detected. (A) Confocal images of M1 and M2 infected with DsRed-expressing *Mtb* H37Rv (cyan) and stained with Lysotracker Deep Red (red), CYTO-ID green (green) and Hoechst 33342 (blue). Shown are images of one representative donor out of four donors. Scale bars annotate 10 μ m. (B) Quantification of the CYTO-ID area per cell and the Lysotracker area per cell of DsRed-expressing *Mtb* H37Rv-infected M1 and M2 after 4h treatment with PARPi (10 μ M) or an equal volume of vehicle control DMSO (0.1% v/v). Data represent the median $\pm$ interquartile range from four different donors. Dots represent the mean from triplicate wells of a single donor. Data are expressed as percentage of control (i.e. DMSO). Differences were significant by RM one-Way ANOVA with Dunnett's multiple comparison test against DMSO. (C) Western immunoblot analysis of autophagic and lysosomal markers. M1 and M2 were infected with *Mtb* H37Rv and treated with PARPi (10 μ M) or an equal volume of vehicle control DMSO (0.1% v/v) for 4h in the presence or absence of bafilomycin A1 (Baf) (10 nM). Shown are blots of one representative donor out of three donors tested and the quantified band intensity is depicted in arbitrary units (AU). Differences were significant by Friedman's test with Dunn's multiple comparison test against DMSO. **p<0.01.



SUPPLEMENTAL FIGURE 4 | Cell viability was assessed by Hoechst/PI staining. M1 and M2 were treated with DMSO or STSP (2.5 μ M) overnight. Microscopy images were taken (left panel) and Hoechst-positive and PI-positive cells were quantified to determine the percentage of live cells (right panel). Shown are the means of three technical replicates from one representative donor, out of four donors tested.

SUPPLEMENTAL TABLE 1 | Coefficient of drug interaction (CID)

	M1		M2	
	RIF (CID $\pm$ SD)	INH (CID $\pm$ SD)	RIF (CID $\pm$ SD)	INH (CID $\pm$ SD)
H-89	1,00 $\pm$ 0,22	1,40 $\pm$ 0,32	0,83 $\pm$ 0,25	0,9 $\pm$ 0,19
A-966492	1,03 $\pm$ 0,20	1,32 $\pm$ 0,17	0,94 $\pm$ 0,28	1,11 $\pm$ 0,21
Niraparib	0,96 $\pm$ 0,19	1,07 $\pm$ 0,27	1,12 $\pm$ 0,34	1,43 $\pm$ 0,52
Pamiparib	0,86 $\pm$ 0,20	1,04 $\pm$ 0,16	0,98 $\pm$ 0,30	0,92 $\pm$ 0,13
Rucaparib	0,92 $\pm$ 0,16	1,17 $\pm$ 0,15	1,06 $\pm$ 0,27	1,04 $\pm$ 0,09

CIDs were calculated based on *Mtb* H37Rv CFUs in M1 and M2 that were treated overnight with PARPi (10 μ M) or an equal volume of vehicle control DMSO (0.1% v/v) in the presence or absence of a suboptimal dose of RIF (0.05 μ g/ml) or INH (0.4 μ g/ml).

SUPPLEMENTAL TABLE 2A | Exposure to PARPi alters the cytokine/chemokine response of M1 and M2 upon *Mtb* infection.

		M1		M2	
		Mock	<i>Mtb</i>	Mock	<i>Mtb</i>
Growth factors	FGF-2	90	110	70	114
	Flt-3L	12	14	10	15
	VEGF	32	32	20	34
Anti-inflammatory cytokines	TGF α	18	15	2	4
	IL-10	7	7	13	135
	IL-1RA	2283	1594	1009	1498
	IL-4	7	4	7	38
Pro-inflammatory cytokines	G-CSF	111	107	71	298
	GM-CSF	11	7	5	17
	IFN α 2	24	30	6	29
	IL-12p40	14	11	7	17
	IL-8	209	473	490	18290
	TNF α	20	62	19	245
Chemokines	MCP-1	352	411	1354	20765
	MIP-1 α	165	255	20	763
	MIP-1 β	153	280	48	1698
	RANTES	10	10	10	40
	MCP-3	61	39	32	50
	Eotaxin	7	7	5	16
	MDC	14472	6439	288	254
	GRO	49	72	119	901
	IP-10	32	47	69	135
	Fractalkine	69	94	84	209

Shown are median cytokine/chemokine levels (in pg/ml) of 8 different in supernatants of M1 and M2 following *Mtb*- or mock-infection overnight. Of the 41 analytes measured, only cytokines/chemokines that were above 10 pg/ml in at least one experimental condition are shown.

SUPPLEMENTAL TABLE 2B | Exposure to PARPi alters the cytokine/chemokine response of M1 and M2 upon *Mtb* infection.

	M1					M2				
	DMSO	A-966-492	Nira-parib	Pami-parib	Ruca-parib	DMSO	A-966-492	Nira-parib	Pami-parib	Ruca-parib
FGF-2	110	70	90	110	70	110	118	110	90	126
Flt-3L	6	12	6	12	14	12	9	14	12	17
VEGF	30	26	30	38	30	34	26	34	27	30
TGF α	14	15	11	13	9	4	3	3	3	2
IL-10	6	7	4	10	7	141	141	87	125	33
IL-1RA	1675	2249	1418	1881	2084	1498	1600	1396	1380	2053
IL-4	24	18	21	24	36	59	71	94	54	60
G-CSF	145	105	80	95	133	325	200	205	209	144
GM-CSF	7	9	5	8	8	16	14	11	11	12
IFN α 2	9	15	8	18	12	27	24	15	12	19
IL-12p40	11	13	11	13	11	13	17	16	13	13
IL-8	473	670	334	671	563	3325	9252	4215	4359	2133
TNF α	62	107	37	177	87	600	545	273	190	249
MCP-1	801	841	379	3387	852	60784		30186	72367	33441
MIP-1 α	255	574	226	2326	867	793	449	280	276	279
MIP-1 β	332	541	211	1403	774	1640	1028	889	840	816
RANTES	13	15	12	14	7	30	19	32	20	7
MCP-3	38	68	42	163	35	45	67	83	348	130
Eotaxin	6	6	6	8	4	13	12	12	12	10
MDC	10253	13992	9075	11425	6517	324	308	299	310	167
GRO	60	81	51	91	57	812	782	723	1108	398
IP-10	36	52	35	48	44	94	150	252	155	81
Fractalkine	120	94	69	167	94	251	165	94	167	210

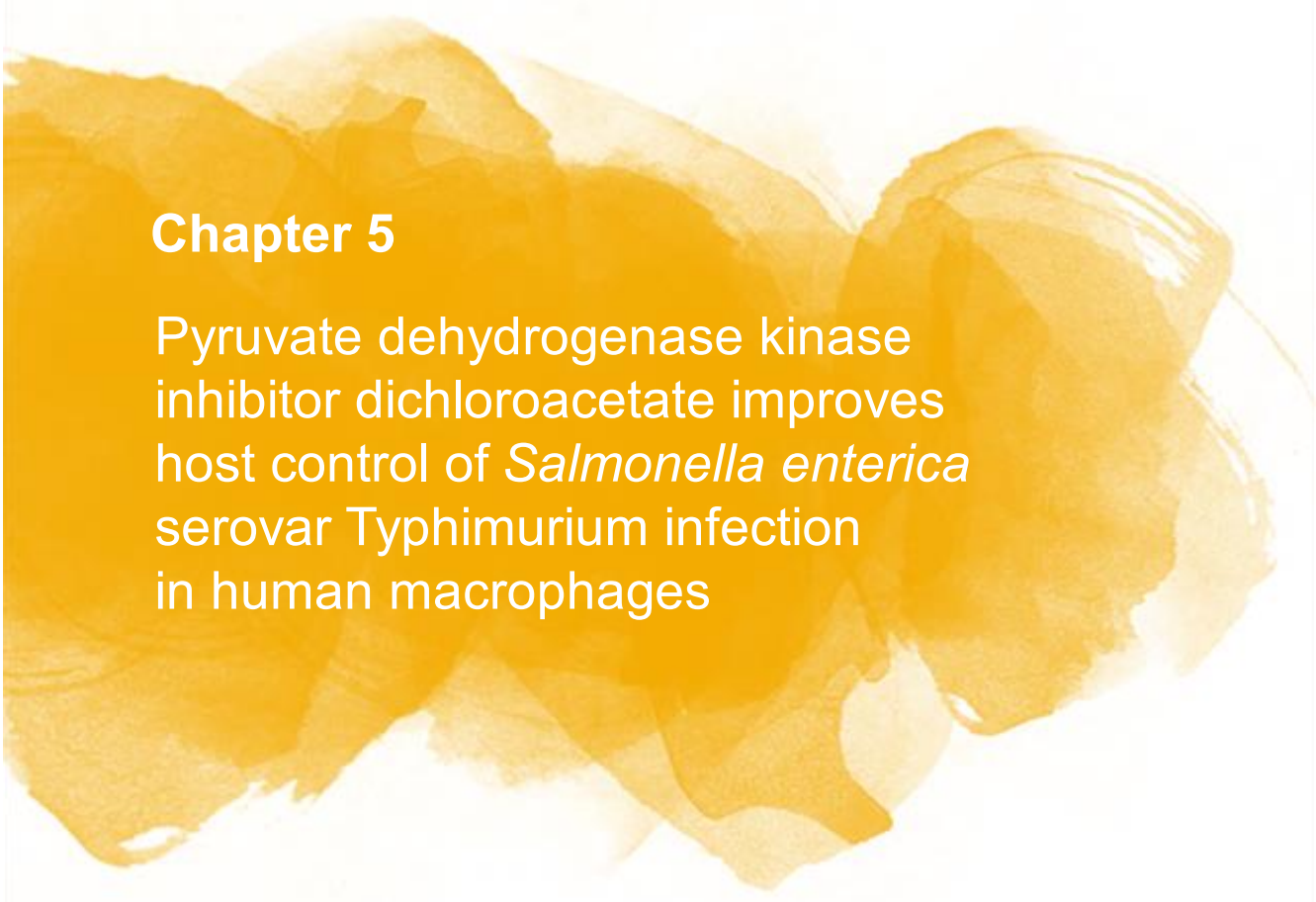
Shown are median cytokine/chemokine levels (in pg/ml) of 4 different donors in supernatants of M1 and M2 in supernatants of *Mtb*-infected M1 and M2 exposed to PARPi (10 μ M) or an equal volume of DMSO (0.1% v/v) overnight. Of the 41 analytes measured, only cytokines/chemokines that were above 10 pg/ml in at least one experimental condition are shown.

SUPPLEMENTAL TABLE 3 | Exposure to PARPi alters surface marker expression on M1 and M2 upon *Mtb* infection.

		M1		M2	
		Mock	<i>Mtb</i>	Mock	<i>Mtb</i>
Pattern recognition	CD14	969	1512	9110	9342
	CD206	28442	32921	13710	17158
	CD209	500	541	1342	1530
Antigen presentation	CD40	14787	19248	7215	25271
	CD80	682	900	948	3167
	CD86	20603	24260	15090	19745
	HLA-DR	74421	95451	41389	55035
Chemotaxis	CD192	9919	12717	6879	8561
	CD197	26	7554	1693	-356
M2-marker	CD163	559	613	2821	1678

	M1					M2				
	DMSO	A-966-492	Nira-parib	Pami-parib	Ruca-parib	DMSO	A-966-492	Nira-parib	Pami-parib	Ruca-parib
CD14	1512	1970	2217	1395	1877	9342	10328	10697	8969	8513
CD206	32921	34622	35967	32864	34976	17158	19556	17143	16462	17941
CD209	541	394	638	530	307	1530	1688	2045	1354	748
CD40	19248	18095	19475	16919	17184	25271	28025	31044	22892	23193
CD80	900	839	867	867	706	3167	3196	3792	3348	2126
CD86	24260	27292	25295	24772	21932	19745	21957	25220	28817	26407
HLA-DR	95451	117827	128625	99274	126451	55035	62550	62234	71647	113397
CD192	12717	13517	12503	10700	8237	8561	9268	9127	7368	6056
CD197	7554	6620	1690	11271	4182	-356	662	1000	1078	-303
CD163	613	896	843	585	624	1678	1719	1949	1583	1481

Shown are median gMFI of 4 different donors in M1 and M2 following *Mtb* or mock infection overnight (top panel) or in *Mtb*-infected M1 and M2 exposed to PARPi (10 μ M) or an equal volume of DMSO (0.1% v/v) overnight (bottom panel).

An abstract, artistic splash of orange and yellow watercolor paint, with various shades and textures, creating a dynamic background for the chapter title.

Chapter 5

Pyruvate dehydrogenase kinase inhibitor dichloroacetate improves host control of *Salmonella enterica* serovar Typhimurium infection in human macrophages

Cassandra L.R. van Doorn, Gina K. Schouten, Suzanne van Veen, Kimberley V. Walburg, Jeroen J. Esselink, Matthias T. Heemskerk, Frank Vrieling and Tom H.M. Ottenhoff

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Abstract

Global increases in the prevalence of antimicrobial resistance highlight the urgent need for novel strategies to combat infectious diseases. Recent studies suggest that host metabolic pathways play a key role in host control of intracellular bacterial pathogens. In this study we explored the potential of targeting host metabolic pathways for innovative host-directed therapy (HDT) against intracellular bacterial infections. Through gene expression profiling in human macrophages, pyruvate metabolism was identified as potential key pathway involved in *Salmonella enterica* serovar Typhimurium (*Stm*) infections. Next, the effect of targeting pyruvate dehydrogenase kinases (PDKs) – which are regulators of the metabolic checkpoint pyruvate dehydrogenase complex (PDC) – on macrophage function and bacterial control was studied. Chemical inhibition of PDKs by dichloroacetate (DCA) induced PDC activation and was accompanied with metabolic rewiring in classically activated macrophages (M1) but not in alternatively activated macrophages (M2), suggesting cell-type specific effects of dichloroacetate on host metabolism. Furthermore, DCA treatment had minor impact on cytokine and chemokine secretion on top of infection, but induced significant ROS production by M1 and M2. DCA markedly and rapidly reduced intracellular survival of *Stm*, but interestingly not *Mycobacterium tuberculosis*, in human macrophages in a host-directed manner. In conclusion, DCA represents a promising novel HDT compound targeting pyruvate metabolism for the treatment of *Stm* infections.

Introduction

The rising prevalence of antimicrobial drug-resistance poses a serious threat to the control of bacterial infectious diseases. Drug-resistance among common bacterial infections continues to cause an estimated mortality rate of 700,000 people annually (1). Two major examples of antibiotic-resistant pathogens are *Mycobacterium tuberculosis* (*Mtb*) and *Salmonella enterica*. The World Health Organization estimated that around 10 million people developed active tuberculosis (TB) in 2017, and that 3.5% of new TB cases and 18% of previously treated TB cases were infected by rifampicin-resistant *Mtb* strains or multidrug-resistant *Mtb*-strains (2). *S. enterica* is a common food-borne pathogen that can cause gastroenteritis and typhoid fever, the latter being responsible for an estimated 200,000 human deaths annually (3). Non-typhoidal *S. enterica* caused invasive disease in 535,000 people in 2017 and two highly invasive drug-resistant *S. enterica* serovar Typhimurium (*Stm*) strains have been described (4–6). Drug-resistance in intracellular pathogens such as *Mtb* and *Stm* is a major concern to global health and poses a critical need for developing new and more effective treatments against such bacteria.

Host-directed therapy (HDT) aims at augmenting host immune responses, mostly as adjunct therapy to currently used antibiotics. HDT may not only provide more effective treatment regimens but may also help shortening current lengthy treatment regimens and thus reducing risk of de-novo resistance mutations as well as reducing antibiotics-induced adverse effects (7). The development of novel HDT, however, requires a better understanding of the biology of intracellular infections and bacterial survival tactics. Recent studies have connected regulation of host defense against bacteria with host metabolic pathways (8). Rapid upregulation of glycolysis in macrophages is a hallmark of *Mtb* infection and LPS stimulation, and resembles the Warburg effect seen in cancer cells, but the mechanisms underlying this metabolic remodeling in bacterially-infected host cells remain poorly understood. Although multiple studies have suggested that rapid upregulation of glycolysis favors host immunity by meeting urgent energy demands required to induce protective immune responses (9–16), more recent studies have shown that tricarboxylic acid (TCA) cycle intermediates, including itaconate, citrate and succinate, are associated with host immune responses against intracellular pathogens as well (17–24). Host metabolic pathways and immune control of intracellular pathogens are thus interconnected in a highly complex manner. The specific immunometabolic pathways underlying macrophage-mediated inflammation and bacterial control remain largely unresolved. This particularly applies to infection-induced metabolic remodeling of human macrophages, which is significantly underrepresented in the literature. In the current study, we therefore modelled host-pathogen interactions in two different, polar human macrophage subsets which are known to respond differently to external stimuli. Classically

activated macrophages (M1) display a pro-inflammatory phenotype and function in response to pathogen-associated molecular patterns (PAMPS) whereas alternatively activated macrophages (M2) display an anti-inflammatory phenotypes and function in response to PAMPS (25, 26). Although the metabolic divergence between M1 and M2 is well described for murine macrophages, surprisingly little is known about human monocyte-derived macrophages (27–29).

Here, we identify the metabolic impact of infections with intracellular bacteria *Stm* and *Mtb* on primary human macrophages and report the prominent effect of the metabolic checkpoint pyruvate dehydrogenase complex (PDC) on *Stm* but not *Mtb* infections. PDC catalyzes the conversion of pyruvate into acetyl-CoA to supply the TCA cycle and is a gate-keeping enzyme regulating the balance between glycolysis and oxidative phosphorylation (OXPHOS). PDC consists of three subunits: pyruvate dehydrogenase (PDH, subunit E1), dihydrolipoamide acetyltransferase (subunit E2) and dihydrolipoamide dehydrogenase (subunit E3). PDH activity is the rate-limiting step during the conversion of pyruvate into acetyl-CoA (30). PDH activity is blocked upon phosphorylation by pyruvate dehydrogenase kinases (PDKs) and restored upon dephosphorylation by pyruvate dehydrogenase phosphatases (PDPs) (31). PDK inhibitors have been developed for treating cancer and type II diabetes (32–34).

In the current study, we report that PDKs are significantly modulated during *Stm* infection in primary human macrophages at the transcriptional level. Dichloroacetate (DCA), a chemical inhibitor of PDKs, activated PDC in M1 and M2 and induced a metabolic shift from glycolysis to OXPHOS in M1. DCA markedly increased production of reactive oxygen species (ROS) by M1 and M2. DCA induced a significant reduction of intracellular *Stm* (but not *Mtb*) in human macrophages, but did not exert a direct antibiotic effect on *Stm* extracellular bacteria, indicating that DCA induces *Stm* control via modulation of host metabolic pathways. In conclusion, DCA represents a promising novel HDT compound targeting pyruvate metabolism for the treatment of difficult to treat *Stm* infections.

Results

Differential Impact of *Stm* Versus *Mtb* Infection-Induced Glycolysis in Primary Human Macrophages

Although LPS stimulation and bacterial infections have been shown to shift cellular metabolism towards a more glycolytic phenotype in various cell types (9–16), a number of studies have reported that human macrophages do not increase glycolysis in response to bacterial stimuli (35, 36). To assess the potential of targeting host metabolic pathways to treat intracellular bacterial infections through HDT, we evaluated the effect of *Stm* and *Mtb* on cellular metabolism in primary human macrophages. Extracellular acidification

rates (ECAR) and oxygen consumption rates (OCR) were measured as indicators of glycolytic and OXPHOS energy phenotypes, respectively, using a Seahorse XFe96 Extracellular Flux Analyzer (experimental outline in Figure 1A). Macrophages were pretreated with bacterial lysates, which we used as a proxy for live infections, due to safety restrictions in the Seahorse XFe96 Extracellular Flux Analyzer laboratory. At baseline, M1 displayed a higher general metabolic activity compared to M2 as evidenced by elevated basal glycolysis and OXPHOS levels (Figures 1B, C; note the different scales on the y-axes). Basal glycolysis was increased in a dose-dependent manner upon 4 hours stimulation with 1 or 10 $\mu\text{g/ml}$ *Stm* or *Mtb* lysate, both in M1 and in M2 compared to unstimulated macrophages (Figures 1B, C and Supplemental Figure 1). OXPHOS was significantly increased upon stimulation with 10 $\mu\text{g/ml}$ *Mtb* lysate in M2, but not upon stimulation with *Stm* lysate. The maximum respiration and glycolytic capacity were significantly increased upon stimulation with 10 $\mu\text{g/ml}$ *Mtb* lysate in M1 and M2 or M2 only, respectively, but not upon stimulation with *Stm* lysate or a low concentration (1 $\mu\text{g/ml}$) of *Mtb* lysate. Calculation of the ECAR/OCR ratios to determine the overall metabolic shift induced in human M1 and M2 following stimulation with *Stm* or *Mtb* demonstrated a bacterially-induced shift towards a more glycolytic phenotype (Figure 1D).

To assess modulation of metabolism by live bacteria, extracellular lactate levels (as an indicator of glycolysis) were determined in macrophages infected with live *Stm* and *Mtb* (Figure 1E). M1 and M2 secreted significantly higher levels of lactate upon *Stm* infection compared to uninfected cells, supporting infection-induced glycolysis. Live *Mtb* infection significantly increased lactate production in M2 compared to uninfected cells, but surprisingly not in M1. The absence of lactate production by *Mtb*-infected M1 corroborates recent findings in mouse bone marrow-derived macrophages (BMDMs) and human monocyte-derived macrophages that *Mtb* is able to actively suppress induction of glycolysis (37, 38).

To confirm induction of a metabolic shift at the transcriptional level, we studied mRNA expression levels of the glycolytic key gene hexokinase 2 (*HK2*) and the TCA cycle key genes succinate dehydrogenase complex flavoprotein subunit A (*SDHA*) and isocitrate dehydrogenase 2 (*IDH2*) in M1 and M2. *HK2* mRNA levels were significantly increased 4 hours post *Stm* infection in M2 (Figure 1F), but were not altered in *Stm*- or *Mtb*- infected M1 (Figure 1B). *SDHA* and *IDH2* mRNA levels were downregulated in *Stm*- and *Mtb*-infected M1 and M2, albeit to different extents, suggesting decreased activity of the TCA cycle pathway. Taken together, our data suggest that *Stm* infections are able to shift human M1 and M2 metabolism towards a more glycolytic phenotype.

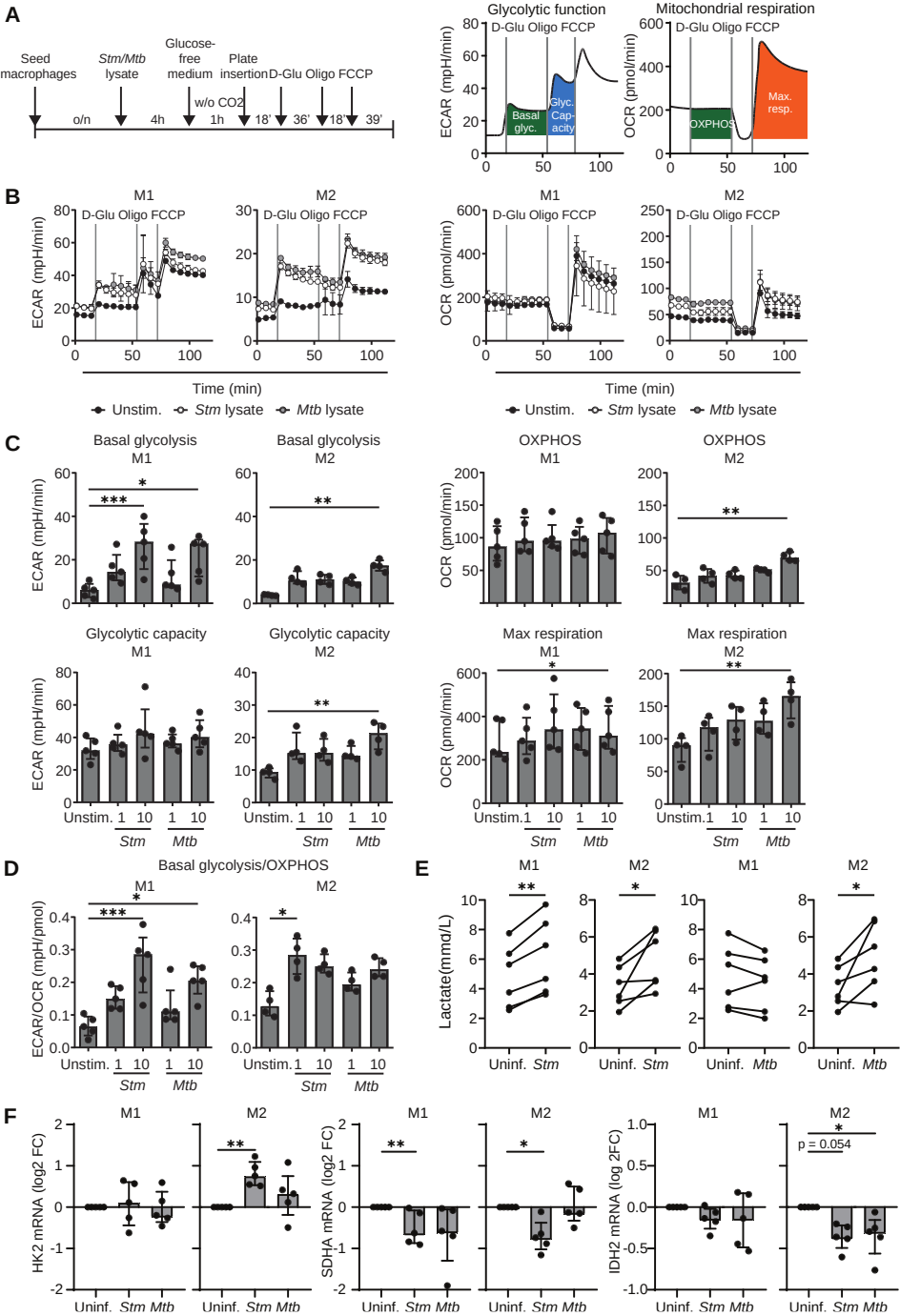


FIGURE 1 | *Stm* and *Mtb* induced a more glycolytic phenotype in human macrophages. (A) Schematic representation of the experimental setup used in (B–D) (left panel) and a representative ECAR and OCR profile indicating basal glycolysis (basal glyc.), glycolytic capacity (glyc. capacity), OXPHOS and maximal respiration (max. resp.) (right panel). M1 and M2 were unstimulated (unstim.) or stimulated with 1 μ g/ml *Stm* or *Mtb* lysate for 4 hours and inserted in the Seahorse analyzer. D-glucose (10 mM), oligomycin (oligo, 1 μ M) and carbonyl cyanide 4-(trifluoromethoxy)-phenylhydrazone (FCCP, 2 μ M) were sequentially injected after 18, 54 and 72 minutes, respectively. (B) ECAR (mpH/min, left panel) and OCR (pmol/min, right panel) profiles of one representative donor out of five (M1) or four (M2) donors analyzed. Data represent the mean $\pm$ S.D. of triplicates. (C, D) Basal glycolysis, OXPHOS, glycolytic capacity, maximum respiration and the ratio between basal glycolysis and OXPHOS (ECAR/OCR) in M1 (left panels) and M2 (right panels). Data represent the median $\pm$ interquartile range of minimally four donors. Differences were significant by RM one-way ANOVA with Dunnett's multiple test correction against the unstimulated control. (E) Lactate production was measured in the supernatant of M1 and M2 that were infected with *Stm* or *Mtb* overnight. Lines connect data points from the same donor, with a total of six donors tested. Significant differences were tested using a paired t-test. (F) *HK2*, *SDHA* and *IDH2* mRNA expression levels were quantified in *Stm*- or *Mtb*-infected M1 and M2 at 4 hours post infection using GAPDH as the housekeeping control gene. Data represent the median $\pm$ interquartile range of five donors. Differences were significant by RM one-way ANOVA with Dunnett's multiple test correction against the uninfected control. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

To study whether the increased glycolysis contributes to host defense, we first treated *Stm*- and *Mtb*-infected M1 and M2 with glycolysis inhibitor 2-deoxy-D-glucose (2-DG), since earlier reports showed that 2-DG increased the burden of *Mtb* in murine BMDMs and in mouse lungs (39, 40). PKA inhibitor H-89 was used as positive control and in agreement with previous findings (41, 42) significantly lowered outgrowth of *Stm*, while being less effective in reducing *Mtb* bacterial load (Figures 2A, B). Surprisingly, treatment of human M1 with 2-DG significantly decreased outgrowth of intracellular *Stm*, but only decreased intracellular *Mtb* in M2 at the highest concentration (5 mM). Importantly, 2-DG did not show cytotoxicity in both macrophage subsets (Figure 2C). The finding that 2-DG treatment did not (M1) or only moderately (M2) affect *Mtb* bacterial load in primary human macrophages contrasts with earlier reports, which concluded that 2-DG treatment increased the burden of *Mtb* in murine BMDMs and in mouse lungs (39, 40). However, 2-DG has recently been described to decrease *Legionella pneumophila* survival independently of inhibition of glycolysis in mouse BMDMs (43) and also, high concentrations of 2-DG have been shown to inhibit not only glycolysis but also OXPHOS in mouse BMDMs (44). Therefore, in order to be able to evaluate the effect of glycolysis independently from the use of 2-DG, we determined bacterial survival after substitution of D-glucose by D-galactose in the cell culture medium. Substitution of D-glucose by D-galactose reduces glycolytic flux and thus could independently validate our findings (44). Indeed, the absence of glucose significantly decreased *Stm* outgrowth in M1, but not M2, and tended to increase intracellular *Mtb* levels (Figures 2D, E). These data suggest that

inhibition of glycolysis impacts *Stm* and *Mtb* in opposite directions; it can inhibit *Stm* outgrowth, while it can promote *Mtb* survival as described (39, 40). Also of importance, these data suggest that pathways other than inhibition of glycolysis are responsible for the antibacterial effect of 2-DG.

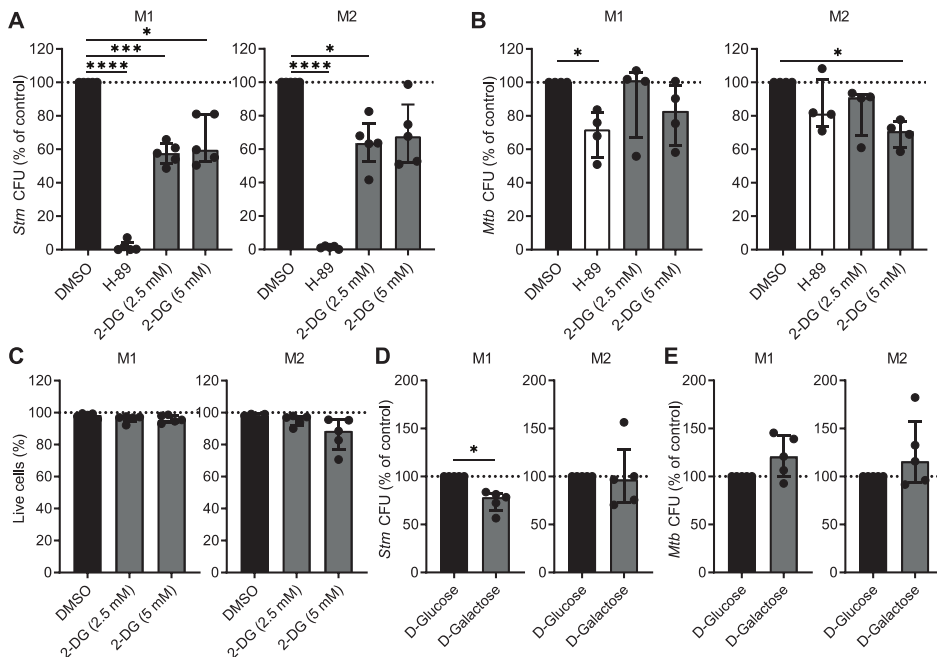


FIGURE 2 | Inhibition of glycolysis decreased intracellular *Stm* and tended to increase intracellular *Mtb* in human macrophages. (A, B) *Stm* and *Mtb* CFUs in M1 (left panels) or M2 (right panels) after treatment with H-89 (10 μ M), 2-DG (2.5 or 5 mM) or vehicle control DMSO (0.1% v/v) overnight. Data represent the median $\pm$ interquartile range of minimally four donors. CFUs are expressed as percentage of DMSO. Differences were significant by RM one-way ANOVA with Dunnett's multiple test correction against DMSO. (C) Percentage of live cells (i.e. Hoechst-positive, PI-negative cells) after overnight treatment with 2-DG (2.5 or 5 mM) or vehicle control DMSO (0.1% v/v) in M1 (left panel) or M2 (right panel). Data represent the median $\pm$ interquartile range of five donors. Differences were tested by RM one-way ANOVA with Dunnett's multiple test correction against DMSO. (D, E) *Stm* and *Mtb* CFUs in M1 (left panels) or M2 (right panels) cultured in medium containing D-glucose (11 mM) or D-galactose (11 mM) for 24 hours. Data represent the median $\pm$ interquartile range of five donors. CFUs are expressed as percentage of DMSO. Significant differences were tested using a paired t-test. * $p < 0.05$, *** $p < 0.001$, **** $p < 0.001$.

Gene Expression Analysis Identifies Pyruvate Dehydrogenase Kinases as Potential Host Targets for HDT During *Stm* and *Mtb* Infections

Next, we aimed to identify metabolic key genes that are modulated during *Stm* and *Mtb* macrophage infections. We performed computational analysis of published RNAseq data by Blischak et al. (45) to study the expression of metabolic genes (i.e. all genes in Reactome pathways 'Glucose metabolism' and 'TCA cycle and respiratory electron transport') in human M2 macrophages infected for 18 hours with *Stm* or *Mtb* (MOI of 5) compared to their time- matched controls (mock infection). *Stm* infection had a more prominent effect on the regulation of glycolytic genes and genes involved in the TCA cycle and respiratory electron transport compared to *Mtb* (Supplemental Figure 2). Two key glycolytic genes, enolase (*ENO*) 2 and *HK2*, were significantly upregulated 18 hours post *Stm* or *Mtb* infection. In contrast, most genes involved in the TCA cycle and respiratory electron transport were downregulated upon *Stm* and *Mtb* infection.

We hypothesized that metabolic genes that are modulated during *Stm* and *Mtb* infections may provide novel targets for HDT. Surprisingly, among the most up- and downregulated metabolic genes during *Stm* and *Mtb* infection, two PDK isoforms were regulated in opposite directions: *PDK4* was strongly downregulated upon *Stm* infection, whereas *PDK1* was strongly upregulated upon *Mtb* infection (Supplemental Figure 3). The differential expression of PDK isoforms is interesting, because PDKs are regulators of the metabolic checkpoint PDC, linking glycolysis with TCA cycle activity and OXPHOS (Figure 3A). More detailed kinetic analysis of the expression levels of all PDK isoforms (*PDK1-4*) and other components of the PDC (*PDP1*, *PDP2*, *PDHX*, *PDHA1* and *PDHB*) showed that several PDK isoforms and catalytic subunits of the PDC were significantly regulated upon infection with *Stm*, in contrast to infection with *Mtb* (Figure 3B), suggesting that pyruvate metabolism was modulated in human macrophages during *Stm* infection.

To confirm and extend the data by Blischak et al. to both M1 and M2 infection models, we measured the expression changes of genes involved in activation of the PDC (i.e.: *PDK1-4*, *PDP1-2*) upon infection with *Stm* and *Mtb* in both M1 and M2. In agreement with the data obtained by Blischak et al., gene expression levels of *PDK2*, *PDK4* and *PDP2* were significantly downregulated in *Stm*-infected human M1 and M2 (Figure 3C). A similar trend could be observed in *Mtb*-infected macrophage subsets, albeit not significant (Figure 3D). Taken together, these data indicate that pyruvate metabolism is markedly modulated during *Stm* infections and to a lesser extent during *Mtb* infections in human M1 and M2, as shown by a downregulation of *PDK2*, *PDK4* and *PDP2* at the transcriptional level.

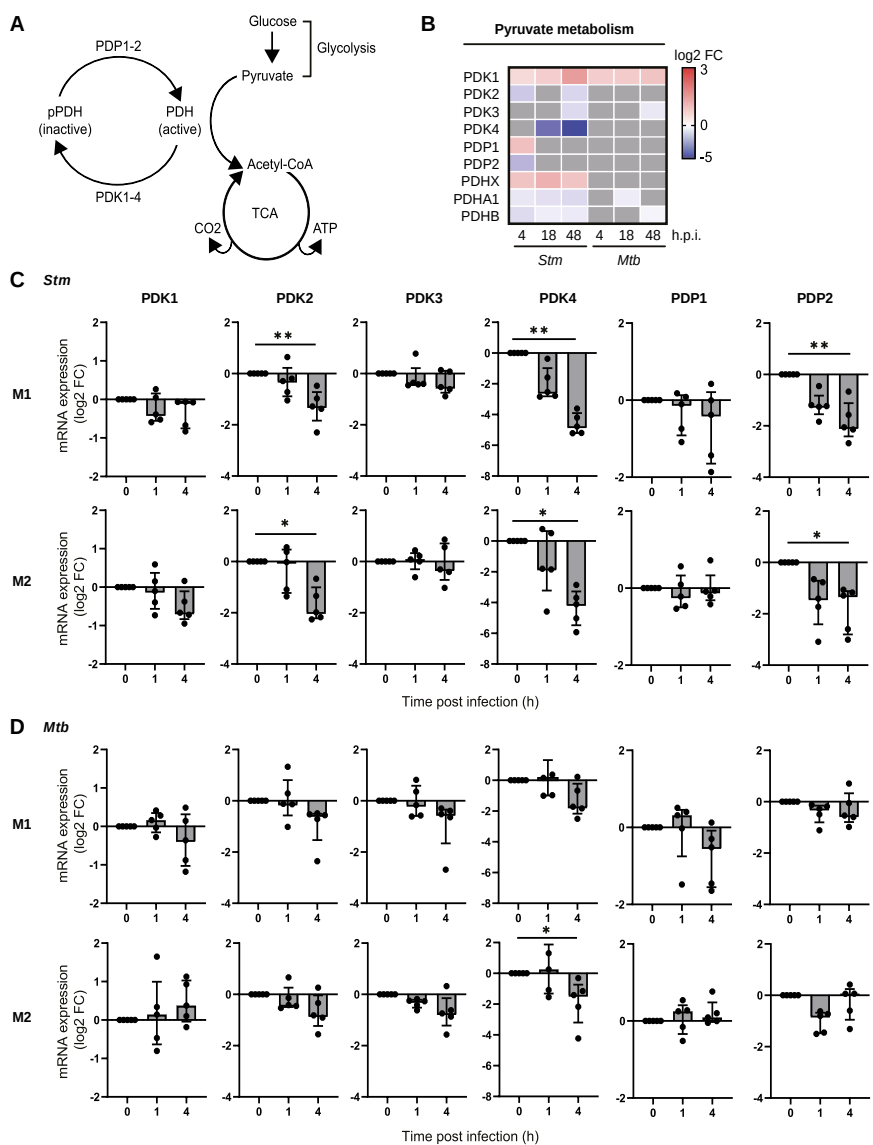


FIGURE 3 | Pyruvate metabolism was regulated during *Stm* infection at the transcriptional level in human macrophages. (A) Schematic representation of the PDC, linking glucose metabolism with the TCA cycle and respiratory electron transport. (B) Differential expression analysis by limma-voom of genes involved in pyruvate metabolism over time in *Stm*- or *Mtb*-infected relative to uninfected M2 macrophages. Significantly up- (red) or downregulated genes (blue) (adjusted *p*-value < 0.05) are indicated on a log₂-FC scale. Grey indicates non-significantly differentially expressed genes. Data obtained from Blischak et al. (45) (C, D) mRNA expression levels were quantified in *Stm*- or *Mtb*-infected M1 (top panels) and M2 (bottom panels) prior to infection (0) or 1 or 4 hours post infection using *GAPDH* as the housekeeping control gene. Data represent the median ± interquartile range of five donors. Differences were significant by RM one-way ANOVA with Dunnett's multiple test correction against the corresponding uninfected control. **p* < 0.05, ***p* < 0.01.

Dichloroacetate Can Be Used as a Tool to Study the Effect of Pyruvate Dehydrogenase Kinases on Intracellular *Stm* and *Mtb* Control in Human Macrophages

To study the effect of pyruvate metabolism during *Stm* and *Mtb* infections in more detail, we aimed to modulate this pathway through chemical inhibition of PDK using dichloroacetate (DCA). To validate inhibition of PDKs as the mechanistic target of DCA, we first examined whether DCA treatment in *Stm* and *Mtb*-infected macrophage subsets resulted in dephosphorylation of PDH at serine residues 293 and 300, which corresponds with increased PDC activation. Pyruvate, a natural PDK inhibitor, was added as a positive control for PDH dephosphorylation. Quantification of the western blot protein band intensities revealed that both DCA treatment and addition of pyruvate resulted in PDH dephosphorylation at serine residues 293 and 300 in *Stm* and *Mtb*-infected M1 and M2 compared to DMSO (Figure 4A). In summary, these data corroborate earlier findings (46, 47) that DCA treatment increases PDC activity, validating PDKs as functional targets of DCA.

Since DCA has been reported to shift metabolism from glycolysis to OXPHOS in various cell lines (32, 46, 48–50), we next evaluated whether DCA could reverse the *Stm*- and *Mtb*-induced metabolic shift towards glycolysis that was predominantly observed in human M1 macrophages (Figure 1C). First, we studied gene expression levels of metabolic key genes (*HK2*, *PDK2*, *PDK4*, *IDH2*, *SDHA*) and downstream targets of succinate accumulation (*HIF1A* and *VEGF*) (19, 22, 51), as an indication of TCA cycle activity, in human macrophages (Figure 4B). DCA did not significantly modify metabolic gene expression at the transcriptional level, but *VEGF* was significantly increased by DCA in *Stm*-infected M1, in accordance with increased TCA cycle activity (Figure 4C). Next, we studied the effect of DCA on OCR and ECAR levels in macrophages that were prestimulated with *Stm* or *Mtb* lysate (Figure 4D). DCA treatment resulted in increased OCR levels and decreased ECAR levels in M1, consistent with a classical DCA-induced shift from glycolysis to OXPHOS (Figure 4E). Strikingly, a shift from glycolysis to OXPHOS could not be detected in M2, indicating that PDC activation does not necessarily lead to glycolytic reprogramming and that this is cell-type specific.

In conclusion, DCA can be used as a chemical tool to study the effect of PDC activation on *Stm* and *Mtb* infections in human macrophages, which is accompanied by metabolic rewiring from glycolysis to OXPHOS in a M1 cell type specific manner.

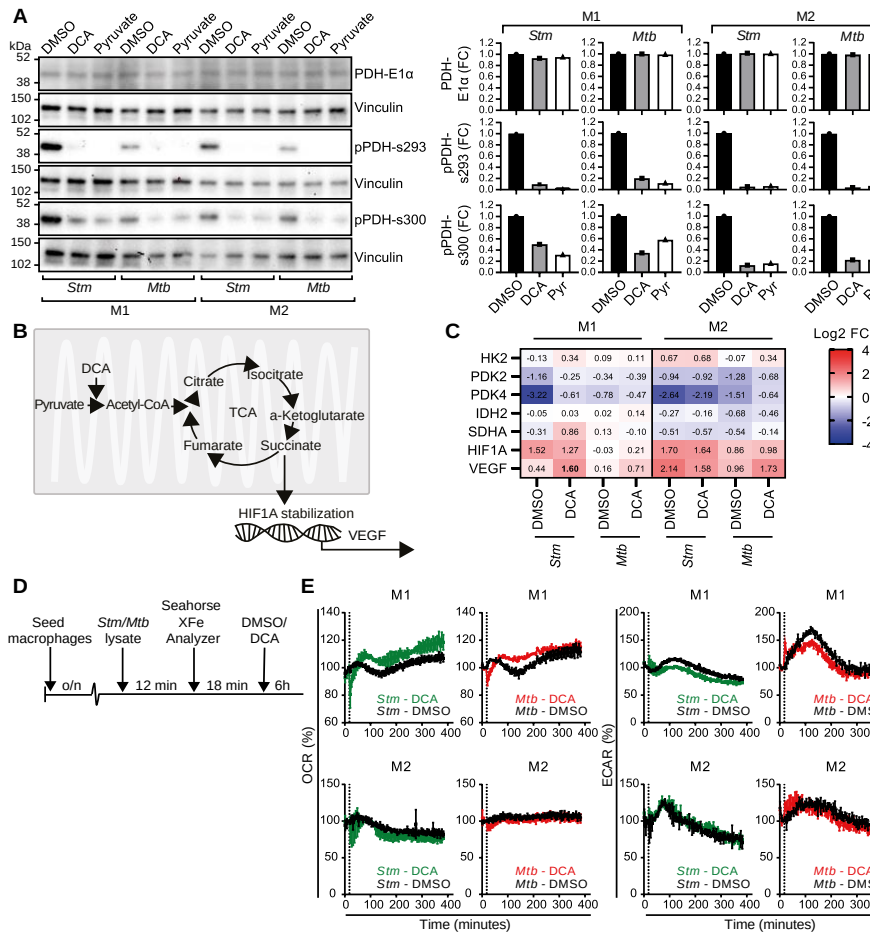


FIGURE 4 | DCA activated the PDC in human macrophages and induced a metabolic shift from glycolysis to OXPHOS in M1. (A) Western blot showing total PDH (PDH-E1α) and PDH phosphorylation at serine residues 293 and 300 at 4 hours post infection in *Stm*- or *Mtb*-infected M1 and M2 treated with DCA (10 mM), pyruvate (10 mM) or vehicle control DMSO (0.1% v/v). Shown is a representative blot from one out of three independent experiments (left panel) and the band intensities were quantified using Fiji/ImageJ, normalized to loading control vinculin and shown as fold change (FC) against DMSO (right panel). (B) Schematic representation of pyruvate metabolism linked with the TCA cycle and HIF-1α stabilization. (C) mRNA expression levels were quantified by Fluidigm qPCR using *GAPDH* as the housekeeping control gene. *Stm*- or *Mtb*-infected M1 and M2 were treated for 4 hours with DCA (10 mM) or vehicle control DMSO (0.1% v/v). Shown are the median ± interquartile range log2FC relative to uninfected macrophages of four donors. Differences were significant by a paired t-test between DMSO and DCA and are shown in bold. (D) Schematic representation of the experimental setup used in (E). (E) The ECAR and OCR were determined over time (0-400 min) in *Stm* or *Mtb*-lysate stimulated human macrophages using the Seahorse XF96 analyzer. M1 and M2 were stimulated with 10 µg/ml *Stm* or *Mtb* lysate for approximately 12 minutes before the first measurement. The vertical dashed line indicates DCA (final concentration 10 mM) or DMSO (at equal v/v) injection at t=18 minutes. Data represent the mean ± standard deviation of triplicates from one representative donor out of four donors.

DCA Treatment Has Minor Impact on Cytokine and Chemokine Production, but Induces ROS Production by Human Macrophages

To study whether PDC activation can modulate effector functions of human macrophages, we assessed the production of 41 chemokines/cytokines by multiplex beads assay. Clearly, *Stm*-infection markedly induced the production of pro- inflammatory cytokines, anti-inflammatory cytokines and chemokines, which contrasted with the lower magnitude changes seen for *Mtb*-infected macrophages (Figure 5A and Supplemental Table 1). DCA treatment did not have any major impact on overall chemokine/cytokine secretion profiles of both M1 and M2, although several individual chemokines and cytokines were significantly modified by DCA in M1 and M2, dependent on the cell-type and the infection type (*Stm* versus *Mtb*) (Figure 5B and Supplemental Table 1).

Since PDC activation by DCA can promote OXPHOS, which is associated with mitochondrial ROS production (46, 52), we next assessed the effect of DCA on ROS production by M1 and M2. Interestingly, DCA significantly induced ROS production in both M1 and M2 and even was a more potent ROS inducer compared to positive control phorbol 12-myristate 13-acetate (PMA) (Figures 5C, D). Surprisingly, ROS production was increased immediately after administration of DCA, even before the first measurement could be taken. ROS production was still observed 1h post DCA stimulation in M1, but reached plateau levels in M2, suggesting that DCA-induced ROS production was halted. DCA-induced ROS production could be partially inhibited by N-acetylcysteine (NAC), which is a scavenger of ROS.

DCA Treatment Induces Intracellular *Stm* Control in Human Macrophages

Since we showed that PDC is a target of interest for HDT and DCA activated PDC, resulting in altered metabolism and increased ROS production, we investigated whether DCA could impact bacterial control by human macrophages. Macrophages were treated with DCA or with H-89 as positive control. As expected, H-89 markedly decreased *Stm* and tended to decrease *Mtb* in M1 and M2 (41). Exposure of *Stm*- and *Mtb*- infected M1 and M2 to 5-20 mM DCA resulted in a profound decrease in *Stm* but not *Mtb* outgrowth (Figure 6A). Importantly, cell viability was not affected (Figure 6B). Interestingly, *Stm* outgrowth was significantly more inhibited by DCA treatment in M1 compared to M2, which is in agreement with the more profound effect of DCA on M1 metabolism (Figure 4E). Furthermore, DCA decreased the percentage of *Stm*-infected M1 and M2 cells compared to DMSO as shown by flow cytometry using DsRed-expressing *Stm* (Supplemental Figure 4A). Interestingly, the effect of DCA on inhibiting intracellular *Stm* outgrowth was already significant after 2 hours of DCA treatment in M2 and after 4 hours in both macrophage subsets, suggesting that DCA induces a host protective response during the early phase of infection (Supplemental Figure 4B).

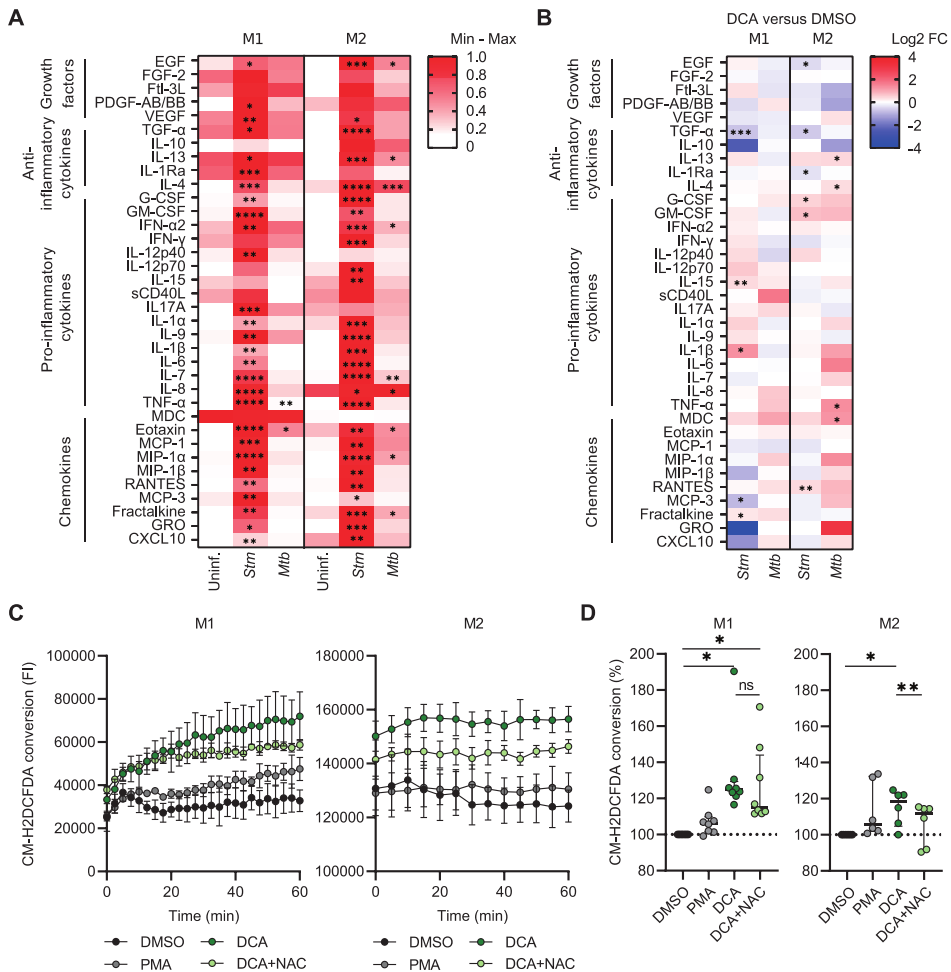


FIGURE 5 | DCA induced ROS production and did not induce major cytokine/chemokine production in human macrophages. (A) Heatmap displaying median cytokine/chemokine levels in the supernatants of uninfected, *Stm*- or *Mtb*-infected M1 and M2 obtained from six donors. Shown are the relative secretion levels (>10 pg/ml) on a white to red color scale per cytokine/chemokine (min=0; max=1). Significant differences were tested by RM one-way ANOVA with Dunnett's multiple comparison test against the corresponding uninfected control. (B) Heatmap displaying median log2 fold change (FC) cytokine/chemokine levels relative to vehicle control DMSO in the supernatants of *Stm*- or *Mtb*-infected M1 and M2 obtained from six donors. *Stm*- or *Mtb*-infected macrophages were treated with DCA (10 mM) or an equal volume of DMSO (0.1% v/v) overnight. Differences were significant by a paired t-test between DMSO and DCA. (C, D) ROS production in M1 (left panel) and M2 (right panel) was measured kinetically for 60 min in cells that were treated with DCA (10 mM), PMA (300 nM) or vehicle control DMSO (0.1% v/v). Data represent the fluorescent intensity (FI) at 522 nm measured kinetically of one representative experiment (C) and the median $\pm$ interquartile range of the area under the curves (AUC) of the FI normalized to control (i.e. DMSO) of minimally six donors (D). Significant differences were tested by RM one-way ANOVA with Tukey's multiple comparison test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.001$.

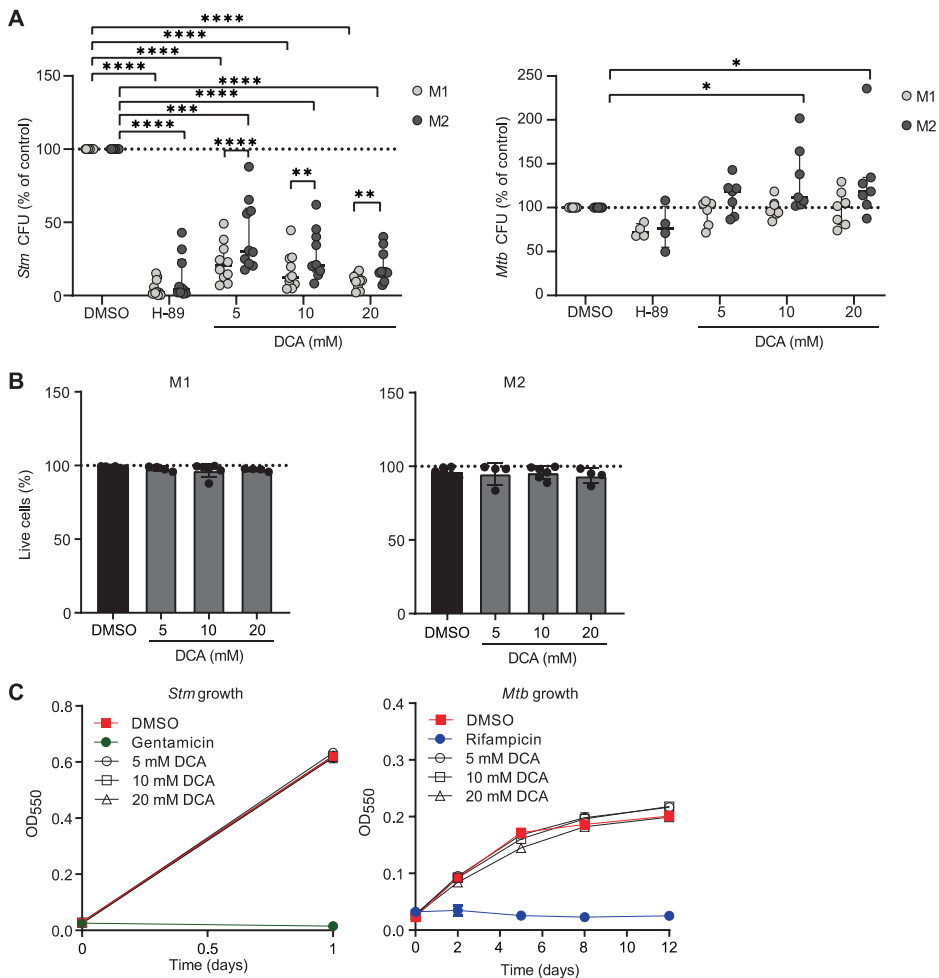


FIGURE 6 | DCA markedly inhibited bacterial outgrowth of *Stm* but not *Mtb*. (A) *Stm*- (left panel) or *Mtb*-infected (right panel) M1 (light grey dots) or M2 (dark grey dots) were treated with H-89 (10 μ M), PDK inhibitor DCA (5–20 mM) or an equal volume of vehicle control DMSO (0.1% v/v) overnight. Data represent the median $\pm$ interquartile range of ten different donors (*Stm*-infection) or seven different donors (*Mtb*-infection). CFUs are expressed as percentage of DMSO. Differences were significant by RM Two-way ANOVA with Dunnett's multiple comparison test to compare DMSO with DCA or with Sidak's multiple comparison test to compare M1 with M2 in each test condition. (B) Percentage of live M1 (left panel) and M2 (right panel) cells (i.e. PI-negative cells) after treatment with DCA (5–20 mM) or vehicle control DMSO (0.1% v/v) overnight. Data represent the median $\pm$ interquartile range of at least four different donors. Differences were tested by RM one-way ANOVA with Dunnett's multiple test correction against DMSO. (C) *Stm* (left panel) and *Mtb* growth (right panel) after addition of DCA (5–20 mM), gentamicin (50 μ g/ml), rifampicin (20 μ g/ml) or vehicle control DMSO (0.1% v/v) to liquid *Stm* (left panel) or *Mtb* (right panel) broth cultures. Data represent the mean optical density (OD) at 550 nm $\pm$ S.D. of triplicates of a representative experiment out of three experiments. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.001.

Not unexpectedly, DCA had little effect on intracellular *Mtb* in M1 and M2 (Figure 6A). Even a prolonged exposure (up to 72h) of *Mtb*-infected macrophages to DCA did not result in reduction of *Mtb* CFU levels (Supplemental Figure 4C), suggesting that *Mtb* modulates host-signaling pathways in a manner different from *Stm*, that cannot be redressed by DCA. Potential direct antibiotic properties of DCA were excluded by adding DCA to *Stm* and *Mtb* cultures in the absence of human cells, showing that DCA acts via host-directed pathways in human macrophages to control intracellular *Stm* (Figure 6C).

Taken together, our data suggest that DCA inhibits intracellular *Stm* infection, but not *Mtb* infection, in a host-directed manner. DCA reduced *Stm* levels rapidly after administration, suggesting that DCA is a potent molecule for treatment against *Stm*.

Discussion

Physiological metabolic modulation of phagocytes is essential to effective host control of intracellular bacterial pathogens (15). Here, we show that human M1 and M2 adapt their metabolic profile upon *Stm* and *Mtb* lysate stimulation and *Stm* infection by increasing glycolysis. We identified metabolic key genes, PDKs, as novel targets for HDT against *Stm* and report DCA as a functional chemical tool to study PDK inhibition in the context of bacterial infections. Furthermore, we report cell-specific effects of DCA on host cell metabolism and chemokine and cytokine production and we show major and rapid effects of DCA on *Stm* outgrowth in infected human macrophages. DCA has been described to induce bacterial clearance in the peritoneum of septic mice (53), and to our knowledge, this is the first report of a host-directed anti-bacterial effect of DCA on *Stm*.

Rapid upregulation of glycolysis is a hallmark of intracellular bacterial infections and is thought to rapidly provide energy to sustain biosynthesis of inflammatory molecules during the initial infection stage (9–16). However, most studies have been performed in mouse models, and the number of studies that assessed induction of glycolysis by bacterial infections in human cellular models is limited. Only few studies have reported that human macrophages do not increase glycolysis in response to LPS and intracellular trypanosomatid parasites (*Leishmania donovani*, *L. amazonensis* and *T. cruzi*) (35, 36). In the current study, M1 and M2 subsets increased their glycolytic metabolism upon infection with *Stm*, as demonstrated by increased extracellular acidification rates and lactate production and reduced succinate dehydrogenase complex subunit A (*SDHA*) mRNA levels. Importantly, and in agreement with findings from others (35, 36), our work does not confirm the reported metabolic divergence between M1 and M2 in mice, where glycolysis is the dominant metabolic pathway in M1 and OXPHOS is the dominant metabolic pathway in M2 in meeting general energy demands (27–29, 54). In fact, in support of our data, LPS stimulation enhanced the glycolytic pathway already after 3 hours

in human M1 and M2 macrophages that were differentiated from monocytes by using M-CSF and IFN- γ or M-CSF, respectively (55). Moreover, glycolysis was induced in human alveolar macrophages upon *Mtb* infection and in human monocytes upon BCG infection (10, 56). Here, we report that stimulation with *Mtb* lysate increased the ECAR/OCR ratio to similar levels as *Stm* lysate. In contrast to infection with live *Stm*, live *Mtb* infection was not able to increase lactate levels in M1, suggestive of decreased glycolytic function in *Mtb*-infected cells compared to *Stm*-infected cells. This supports the findings by others that live *Mtb* may actively suppress the glycolytic pathway in M1 (37, 38).

Inhibition of glycolysis by 2-DG inhibited *Stm* but not *Mtb* outgrowth in our macrophage model. Importantly, our data in primary human macrophages are in contrast with published data from mouse BMDMs in which inhibition of glycolytic flux by 2-DG or galactose reduced IFN- γ -dependent control of intracellular *Mtb* (39). Moreover, mice treated *in vivo* with 2-DG had higher *Mtb* bacterial burdens in their lungs after 14 days (40). Although 2-DG is classically reported to inhibit glycolysis, it also inhibits OXPHOS when used in high concentrations that are similar to the ones in our study (up to 10 mM) (44). Furthermore, the inhibitory effect of 2-DG on *Stm* and *Mtb* infection could be dependent on other pathways than its impairment of the glycolytic pathway or OXPHOS, as has already been suggested for *Legionella pneumophila* (43). In agreement with others, we thus propose caution in the interpretation of data generated using 2-DG in the context of glycolysis (44, 57, 58). Alternatively, substitution of glucose for galactose can provide a more reliable method to inhibit glycolysis (44). In our work substitution of D-glucose for D-galactose tended to increase intracellular *Mtb*, in agreement with reported data in mouse BMDM (39), and in line with the hypothesis that glycolysis is required for protective host responses against *Mtb*. Importantly, substitution of D-glucose for D-galactose significantly decreased intracellular levels of *Stm*, suggesting that the classical “host-protective” role of glycolysis during bacterial infections does not apply to *Stm* in human macrophages.

PDKs are considered gate-keeping kinases between glycolysis and OXPHOS and chemical inhibition of PDKs by DCA resulted in a significant reduction of intracellular *Stm* outgrowth in human macrophages. DCA is a potent small molecule in clinical use for the treatment of lactic acidosis and metabolic disorders (59) and has gained serious interest as anti-cancer therapeutic in the past decade (60). DCA has passed phase I/II toxicity testing in humans, which can accelerate the translation towards to clinic application for other diseases. Administration of DCA displayed a broad spectrum regarding toxicity and safety, ranging from well-tolerance (61) and common gastrointestinal side-effects (62), to severe side-effects like peripheral neuropathy (63, 64). Despite reported toxicities, DCA has not lost interest as potential therapeutic, and severe side-effects may be suppressed

with adjuvant therapy (65). Tolerance of DCA in patients infected with *Salmonella* remains to be determined and is crucial for its applicability as HDT compound for the treatment of *Salmonella* infections, which has a mild disease course in most patients, but can be persistent and even lethal in others (66, 67). Building upon this potential, we have identified PDKs as potential novel host targets for HDT to treat *Stm* infections. Of note in this context, several novel, potentially safer, DCA derivatives have been synthesized recently (59).

DCA classically induces a metabolic shift from glycolysis to OXPHOS, which was reported in cancer cell lines (32, 46, 48–50) and mouse embryonic stem cells (68). Similarly, DCA significantly decreased ECAR levels in human macrophages differentiated with M-CSF or M-CSF with IFN- γ (55). Here, we report a similar result using DCA in M1 stimulated with *Stm* or *Mtb* lysate, but not in M2. Differences in ECAR levels between M-CSF-derived macrophages published by Chiba et al. and our M-CSF-derived M2 may be explained by differences in DCA concentrations tested (20 mM versus 10 mM in our study), or by the use of pre-stimulation with *Stm* or *Mtb* lysate in our study. In addition to decreased ECAR levels, we observed increased OCR levels immediately after DCA treatment in M1, indicative of a metabolic shift from glycolysis to OXPHOS. Interestingly, we observed increased levels of *VEGF* mRNA levels in *Stm*-infected M1 and not in M2. Since succinate has been associated with HIF-1 α stabilization, we hypothesize that the increased *VEGF* mRNA levels are a consequence of increased flux into the TCA cycle leading to succinate accumulation. Thus, importantly, we show that DCA induces cell-type specific responses, but not general metabolic rewiring in all cell types. Since DCA-induced metabolic effects were only observed in M1 and not in M2, we propose that improved control of *Stm* was not caused by a metabolic shift from glycolysis to OXPHOS. While not investigated here, DCA treatment may have increased or decreased accumulation of specific TCA cycle metabolites that modulate innate immune responses of macrophages to *Stm*, such as succinate (69), or may have deprived the cells from host carbon sources that can be exploited by *Stm*, for instance lactate (70).

In previous studies, DCA induced the functional polarization of murine macrophages towards an anti-inflammatory phenotype based on cytokine production profiles (71, 72). Similarly, in human cells, DCA decreased IL-1 β and IL-10 levels in LPS-stimulated human THP-1 cells (73) and IL-6 levels in LPS-stimulated human macrophages (55). In contrast with these studies, we did not observe significant DCA-inhibiting effects on IL-1 β , IL-10 and IL-6 secretion by *Stm*- or *Mtb*- infected human macrophages, possibly because infection alone already strongly skewed the cytokine response (Figure 5A). Generally, we observed that the effect of DCA on chemokine/ cytokine secretion was dependent on both cell-type (M1 versus M2) and infection (*Stm* versus *Mtb*). We therefore conclude that, based on

their cytokine secretion profile, DCA was not able to (re)polarize *Stm*- or *Mtb*-infected human M1 and M2 towards an anti-inflammatory phenotype.

Potential mechanism by which DCA induces *Stm* control in human macrophages is through increasing ROS production, as DCA clearly induced ROS in both M1 and M2. In agreement with our data, DCA increased intracellular ROS production by tumor cells, head and neck cancer cells and multiple myeloma cells (46, 52, 74). Although ROS production appears a host strategy to cope with *Stm* (75–77), it can also promote *Stm* survival, and thus ROS acts as a double-edged sword in the perspective of the host and *Stm* (78). While DCA-induced ROS production has been suggested to originate from increased OXPHOS activity in the mitochondria (46, 74), we cannot not exclude other sources of ROS production based on our data. Increased ROS production in M2, despite no detectable changes in OXPHOS in these cells, in fact might suggest that ROS may have originated from other sources, for instance NADPH oxidase-dependent ROS production of phagosomal origin (79). Given that the type III secretory system of *Salmonella* interferes with host NADPH oxidase trafficking to the phagosome (as reviewed in 80) to evade ROS-induced killing by phagocytes, we speculate that NADPH localization may be restored by DCA treatment. This effect of DCA on NADPH oxidase localization and the effect of ROS production on intracellular *Stm* control requires future investigation.

In the current study, we showed that PDK expression is modulated during *Stm* infections and can be targeted by DCA to improve host control against *Stm* infections. Furthermore, we evaluated effects of DCA on *Stm* from a host perspective. Whether the effect of DCA on *Stm* control is caused by limited pyruvate access as carbon source remains to be determined, but may explain why DCA did not decrease intracellular *Mtb* levels. Together, our data highlight differences in survival of two pathogenic bacteria that can use the same host niche, and show the clinical potential of DCA as novel HDT treatment against *Stm*.

Materials and Methods

Reagents and Antibodies

Dimethyl sulfoxide (DMSO), 2-deoxy-D-glucose (2-DG), D- (+)-glucose, D-(+)-galactose, sodium dichloroacetate (DCA), sodium pyruvate, sodium L-lactate, H-89 dihydrochloride hydrate (H-89), and phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands). Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) was obtained from Seahorse Bioscience (Billerica, MA, USA). FITC Anti-human CD14 monoclonal (M5-E2) and A647 anti-human CD163 monoclonal (RM3/1) were purchased from Sony Biotechnology Inc. (San Jose, California, USA). PE anti-human CD11b monoclonal (ICRF44) was obtained from BD Bioscience (Vianen, The Netherlands).

Anti-human PDH-E1a (pSer293) and anti-human PDH-E1a (pSer300) polyclonal antibodies were purchased from Merck Millipore (Amsterdam, The Netherlands). Anti-human vinculin monoclonal (hVIN-1) was purchased from Sigma-Aldrich and anti-human PDH monoclonal (9H9AF5) from Thermo Fisher Scientific (Landsmeer, The Netherlands). Secondary stabilized peroxidase conjugated antibodies goat anti- rabbit IgG (H+L) and goat anti-mouse IgG (H+L) were purchased from Thermo Fisher Scientific.

Cell Culture

Primary human macrophages were obtained as described earlier (25). Briefly, human peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats obtained from healthy donors with informed consent by density gradient centrifugation over Ficoll/amidotrizoat (Pharmacy, LUMC, The Netherlands). CD14⁺ monocytes were isolated by magnetic cell sorting using anti-CD14-coated microbeads (Miltenyi Biotec, Auburn, CA) and cultured at 37°C/5% CO₂ in Gibco Roswell Park Memorial Institute (RPMI) 1640 medium (Thermo Fisher Scientific) supplemented with 10% FBS, 2 mM L-Alanyl-L-Glutamine (PAA, Linz, Austria), 100 units/ml penicillin, 100 µg/ml streptomycin and either 5 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF, Thermo Fisher Scientific) to promote M1-differentiation or 50 ng/ml macrophage colony-stimulating factor (M-CSF, R&D Systems, Abingdon, United Kingdom) to promote M2-differentiation. After 6 days of differentiation, macrophages were detached by trypsinization and gentle scraping, counted and seeded for downstream application in RPMI 1640 medium (10% FBS and 2 mM L-Alanyl-L-Glutamine). For experiments, cells were incubated overnight at 37°C/5% CO₂. In parallel, M1 and M2 macrophage phenotypes were validated based on surface marker expression (M1: CD14^{low}, CD163^{low}, CD11b^{high}; M2: CD14^{high}, CD163^{high}, CD11b^{low}) by flow cytometry based on cytokine production (IL-10 and IL-12) following overnight LPS stimulation (100 ng/ml) as described before (81). Cells were plated on round-bottom 96 wells plates in a concentration of 75,000 cells/well in RPMI 1640 and washed in 150 µl PBS/0.1% BSA. Fc receptors were blocked by adding 50 µl of 5% human serum (Sanquin, Amsterdam, the Netherlands) for 10 minutes. Cells were washed and stained with antibodies against CD14, CD163 and CD11b for 30 minutes at 4°C in the dark, washed twice and fluorescence intensity was measured on a Accuri™ C6 flow cytometer (BD Bioscience).

Bacterial Culture

DsRed-expressing-*Salmonella enterica* serovar Typhimurium (*Stm*) strain SL1344 (42) was recovered from frozen stock and cultured at 37°C in Difco™ Luria-Bertani (LB) broth (BD Bioscience) containing 100 µg/ml ampicillin (Sigma-Aldrich). DsRed-expressing-*Mycobacterium tuberculosis* H37Rv (42) was cultured at 37°C in Difco™ Middlebrook 7H9 broth (BD Bioscience, Vianen, The Netherlands) containing 10% ADC (Becton Dickinson,

Breda, The Netherlands), 0.5% Tween-80 (Sigma-Aldrich) and 2% Glycerol (Sigma-Aldrich). *Stm* and *Mtb* lysates were generated by harvesting log-phase liquid *Stm* and *Mtb* cultures. Cultures were washed twice and resuspended in PBS. 1 ml glass beads (0.1 mm, BioSpec Products, Breda, The Netherlands) were added to 1 ml bacterial suspension in PBS and lysates were generated using the Mini-BeadBeater-1 (BioSpec Products). After 5 minutes of incubation, cell lysates were transferred to a 1.5 ml Eppendorf tubes and protein concentration was quantified by Pierce™ BCA protein assay kit according to manufacturer's instructions (Thermo Fisher Scientific).

Seahorse Real-Time Extracellular Flux Analysis

Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were determined as a measure of mitochondrial respiration and glycolysis, respectively, by using the Agilent Seahorse XFe96 Extracellular Flux Analyzer (Seahorse Bioscience) according to manufacturer's instructions and as described before (82). Briefly, cells were plated in a Seahorse XF cell culture microplate at a density of 30,000 cells/well and incubated overnight at 37°C/5% CO₂ in RPMI 1640 medium (10% FBS, 2 mM L-Alanyl-L-Glutamine). Cells were stimulated with 1 or 10 µg/ml *Stm* or *Mtb* lysate and incubated for 4 hours at 37°C/5% CO₂. Plates were washed with glucose-free RPMI 1640 medium (Sigma-Aldrich) containing 5% FBS and incubated for 1h at 37°C without CO₂. To determine the effect of chemical compounds on OCR and ECAR, cells were stimulated with 10 µg/ml *Stm* or *Mtb* lysate and inserted in the Seahorse XFe96 Analyzer directly. After insertion into the Seahorse XFe96 Analyzer, wells were sequentially injected with final concentrations of 10 mM D-glucose with or without chemical compounds, 1 µM oligo (ATP synthase inhibitor) and 2 µM FCCP (mitochondrial uncoupling agent) after 18, 54 and 72 minutes, respectively. Basal glycolysis was calculated as the difference between lowest ECAR measurement pre-D-glucose injection and highest ECAR measurement post-D-glucose injection. The glycolytic capacity was calculated as the difference between lowest ECAR measurement pre-D-glucose injection and highest ECAR measurement post-oligo injection. OXPHOS was calculated as the difference between highest OCR measurement pre-oligo injection and lowest OCR measurement post-oligo injection. Maximal respiration was calculated as the difference between lowest OCR measurement pre-FCCP injection and highest OCR measurement post-FCCP injection. Basal glycolysis was divided by OXPHOS to calculate ECAR/OCR ratios.

Macrophage Infection

Ongoing *Stm* cultures were diluted one day before infection and again at 2-3 hours prior to infection to reach log phase (OD600 between 0.4-0.6) during infection. *Mtb* cultures were diluted one day prior to infection to reach log phase (OD600 between 0.4-0.6) during infection. Cells were infected with *Stm* or *Mtb* with a multiplicity of infection (MOI) of 10.

Accuracy of the MOI was validated by plating serial dilutions of the *Stm* inoculum on Difco™ LB agar plates (BD Bioscience) or the *Mtb* inoculum on Difco™ Middlebrook 7H10 agar (BD Bioscience) plates containing 10% OADC (Becton Dickinson) and 5% glycerol. Cells seeded 1 day prior to infection in flat-bottom 96-well plates were inoculated with 100 µl bacterial suspension and cells seeded in flat-bottom 24-well plates with 500 µl bacterial suspension in RPMI 1640 (10% FBS, 2 mM L-Alanyl-L-Glutamine). Plates were centrifuged for 3 minutes at 800 rpm and incubated for 20 minutes for *Stm* infections and 1 hour for *Mtb* infections at 37°C/5% CO₂. Extracellular bacteria were removed by washing and incubation with fresh RPMI 1640 (10% FBS, 2 mM L-Alanyl-L-Glutamine) containing 30 µg/ml gentamicin sulphate (Lonza BioWhittaker, Basel, Switzerland) for 10 minutes and cells were incubated in gentamicin sulphate-containing medium (5 µg/ml) in the presence or absence of chemical compounds or an equal amount of vehicle control DMSO (0.1% v/v) for 4 hours or overnight at 37°C/5% CO₂.

Lactate Assay

Cell supernatants were harvested and kept at -20°C until further use. 10 µl of 100 mM sodium l-lactate standard or sample was added in triplicate to a flat bottom 96-well plate (Greiner Bio-One, Alphen a/d Rijn, The Netherlands). 200 µl of reaction mix (0.74 mM NAD, Roche Applied Science; 0.4 mM glycine, Sigma-Aldrich; 0.4 M hydrazine hydrate, Sigma-Aldrich) and 2 µl of three times diluted L-Lactate Dehydrogenase (LDH) from rabbit muscle (Roche Applied Science, Bayern, Germany) was added to each well to allow conversion of lactate to pyruvate by LDH and trapping of the newly formed pyruvate: Lactate + NAD⁺ ↔ Pyruvate + NADH + H⁺. Plates were incubated on a shaker for 90 minutes at RT and NADH levels were measured spectrophotometrically at 340 nm using a SpectraMax i3x plate reader (Molecular Devices, CA, USA) before and after incubation as an indicator of lactate production.

CFU Assay

Intracellular bacterial burden was determined by colony forming unit (CFU) assays as described previously (81). Cells were washed once with PBS and lysed in 0.05% sodium dodecyl sulfate (SDS) solution (Thermo Fisher Scientific). Serially diluted cell lysates were plated on LB agar (*Stm*) or on 7H10 agar containing 10% OADC and 5% Glycerol (*Mtb*). Plates were incubated at 37°C and CFUs were counted from triplicate wells.

Cellular Toxicity Assay

The number and percentage of dead cells based on plasma membrane integrity of the adherent cell population was quantified by analysis of microscopy images. Cells in 96-well flat bottom plates (30,000 cells/well) were stained with 2 µg/ml propidium iodide (PI, Sigma-Aldrich) and 2 µg/ml Hoechst 33342 (H3570, Sigma-Aldrich) in 40 µl/well phenol

red-free RPMI 1640 (Sigma-Aldrich) supplemented with 10% FBS and 2 mM L-Alanyl-L-Glutamine and incubated for 5 minutes at room temperature (RT). Cells were imaged using a Leica AF6000 LC fluorescence microscope (Leica Microsystems, Wetzlar, Germany) combined with a 10x dry objective. Total and dead cell numbers were quantified by respectively counting the nuclei and the number of PI-positive cells using Fiji/ImageJ software (83).

Cell-Free Bacterial Growth Assay

Compounds were diluted in Difco™ LB broth or in Difco™ Middlebrook 7H9 broth and added (100 µl/well) to 100 µl/well bacterial culture (OD600 of 0.1) in flat bottom 96-well plates. Gentamycin (50 µg/ml) and rifampicin (20 µg/ml) were added as positive controls to inhibit *Stm* and *Mtb* growth, respectively. Absorbance was measured directly after plating and after 16-18 hours for *Stm* growth and at day 2, 5, 8 and 12 for *Mtb* growth at 550 nm on a Mithras LB 940 plate reader (Berthold Technologies, Bad Wildbad, Germany).

Fluidigm Real-Time Quantitative Polymerase Chain Reaction (qPCR)

300,000 cells/well in a flat bottom 24 wells plate were lysed in 350 µl TRIzol™ reagent (Thermo Fisher Scientific). RNA was isolated according to manufacturers' instructions and diluted to 50 ng/µl. cDNA and 14-cycle preamplification was performed according to Fluidigm protocols (Biomark Fluidigm, South San Francisco, CA, United States). Briefly, cDNA was synthesized by addition of 1 µl reverse transcription master mix to 3 µl of RNase-free water and 1 µl of 50 ng/µl RNA per sample, and incubated using the following thermal protocol: 5 min at 25°C, 30 min at 42°C, 5 min at 85°C and then stored at -20°C until use. For preamplification, 1.25 µl of cDNA was added to 1 µl of Preamp Fluidigm Master Mix, 1.25 µl of Pooled TaqMan primer mix and 1.5 µl of RNase-free water per sample, and incubated using the following thermal protocol: 2 min at 85°C, 14 cycles of 15s at 95°C and 4 min at 60°C and stored at -20°C until use. For quantitative real-time PCR reactions, 1.35 µl preamplified cDNA, 1.5 µl 2X Taqman Gene Expression PCR Master Mix and 0.15 µl 20X GE Sample Reagent was used per reaction. Flex Six fluidics chips were primed with control line fluid and loaded with 3 µl preamplified samples in the appropriate inlets. All Real-Time PCR reactions were performed in the BioMark real-time PCR system using the GE FLEXSix Standard v1 thermal protocol. Cycle threshold (Ct) values were calculated using BioMark Real-time PCR Analysis software. Changes of expression values were calculated as the log2 Fold Change (FC) between the target gene and the reference gene GAPDH. The following FAM-MGB Taqman primer sets were used: *GAPDH* (Hs02758991_g1), *PDK1* (Hs01561847_m1), *PDK2* (Hs00176865_m1), *PDK3* (Hs00178440_m1), *PDK4* (Hs01037712_m1), *PDP1* (Hs01081518_s1), *PDP2* (Hs01934174_s1), *HK2* (Hs00606086_m1), *SDHA* (Hs00188166_m1), *IDH2* (Hs00953879_m1), *HIF1A* (Hs00153153_m1) and *VEGFA* (Hs00900055_m1).

Western Blot Analysis

Cells (300,000 cells/well in 24-wells plates) were lysed using 100 µl/well EBSB buffer (10% v/v glycerol, 3% SDS, 100 mM Tris-HCl, pH 6.8) supplemented with cOmplete™ EDTA-free protease inhibitor cocktail (Sigma-Aldrich) and phosphatase inhibitor (PhosSTOP EASYpack Sigma-Aldrich) in the concentration of one tablet each per 10 ml. Samples were boiled for 10 minutes at 95°C and stored at -20°C until use. Sample concentrations were determined using Pierce™ BCA protein assay kit (Thermo Fisher Scientific) according to manufacturer instructions and diluted to equal concentrations in Laemmli sample buffer (Biorad) containing 10% b-mercaptoethanol (Sigma-Aldrich). 12.5 µl of sample per lane was loaded on a 15-well 4–20% Mini-PROTEAN® TGX™ Precast Protein Gel (Bio-Rad Laboratories, Veenendaal, the Netherlands) and Amersham ECL Rainbow Molecular Weight Marker (Sigma-Aldrich) was added as reference. Proteins were transferred to methanol-activated Immun-Blot PVDF membranes (Biorad) in Tris-glycine buffer (25 mM Tris, 192 mM glycine and 20% methanol). Membranes were blocked for 1 hour in polysorbate 20 tris-buffered saline (TTBS) supplemented with bovine serum albumin fraction V (BSA, 5% w/v, Roche Diagnostics, Almere, The Netherlands) and incubated with the indicated antibodies in 5% BSA/TTBS overnight at 4°C. Membranes were washed for 45 minutes in TTBS and stained with secondary antibodies in 5% BSA/TTBS for 2 hours at RT. Membranes were washed for 30 minutes with TTBS prior to revelation using enhanced chemiluminescence (ECL)™ Prime Western Blotting System reagent (GE Healthcare, Hoevelaken, The Netherlands). Imaging was performed on a ChemiDoc XRS+ (Bio-Rad). Protein bands were quantified using ImageJ/ Fiji software (83) and normalized to vinculin.

Reactive Oxygen Species (ROS) Assay

Procedure was performed as described previously (81). Cells (40,000 cells/well) were seeded in black ultra-thin clear flat bottom 96-well plates (Corning) in RPMI 1640 (10% FBS, 2 mM L-Alanyl-L-Glutamine) and incubated overnight at 37°C/ 5% CO₂. Cells were washed with 150 µl PBS/well and RPMI 1640 without phenol red was added and background fluorescence was measured using a SpectraMax i3x plate reader (l ex 493 nm and l em 522 nm) at 37°C. Cells were washed with 150 µl PBS/well. 50 µl/well of 5 µM CM-H2DCFDA, a general ROS probe, in RPMI 1640 without phenol red was added for 30 minutes at 37°C/5% CO₂. Cells were washed with 100 µl PBS/well and 100 µl/well RPMI 1640 without phenol red was added containing DMSO (0.1% v/v), DCA (10 mM) or PMA (300 nM) as positive control. ROS production was measured kinetically for 60 minutes at 37°C by measuring fluorescence at 522 nm using the SpectraMax i3x.

Cytokine and Chemokine Secretion by Enzyme-Linked Immuno Sorbent Assay (ELISA) and Multiplex Beads Assay

Cell supernatants were collected and sterilized by centrifugation using a 96-well filter plate containing a 0.2 μ m PVDF membrane (Corning). IL-12p40/p70 production was quantified using a human IL-12p40/p70 Enzyme-Linked Immuno Sorbent Assay (ELISA) kit (Thermo Fisher Scientific) according to manufacturer's instructions. Secretion levels of forty-one cytokine/chemokine analytes were quantified using the Milliplex Human Cytokine/Chemokine magnetic bead premixed 41-plex kit (Millipore Billerica, MA, USA) according to manufacturer's instructions. The following analytes were measured on a Bio-Plex 100 with Bio-Plex Manager™ software v6.1 (Biorad): sCD40L, EGF, FGF-2, Flt3 ligand, Fractalkine (CX3CL1), G-CSF, GM-CSF, GRO (CXCL1), IFN- γ , IFN- α 2, IL-1 α , IL-1 β , IL-1RA, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8 (CXCL8), IL-9, IL-10, IL-12p40, IL-12p70, IL-13, IL-15, IL-17 α , IP-10 (CXCL10), MCP-1 (CCL2), MCP-3 (CCL7), MDC (CCL22), MIP-1 α (CCL3), MIP-1 β (CCL4), PDGF-AB/BB, RANTES (CCL5), TGF- α , TNF- α , TNF- β , VEGF, Eotaxin (CCL11) and PDGF-AA. Cytokines that were produced in quantities lower than 10 pg/ml in all treatment conditions were excluded from analysis.

Statistical Analysis

Data were tested with a paired sample t-test when comparing two groups and RM one-way ANOVA followed by Dunnett's multiple comparison test, unless mentioned otherwise, when comparing three or more groups. Statistical tests were considered significant when $p < 0.05$ at 95% confidence interval. All statistical analyses were carried out using GraphPad Prism 8 software (Graphpad Software, San Diego, CA, USA).

Data Availability Statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics Statement

The studies involving human participants were reviewed and approved by Institutional Review Board of the Leiden University Medical Center, The Netherlands. The patients/participants provided their written informed consent to participate in this study.

Author Contributions

TO and CD devised the project and the main conceptual ideas. MH and FV contributed to the design and implementation of the research. CD, GS, JE, SV, and MH performed the experiments and analyzed the experimental data. KW contributed to preparation of materials. CD and TO wrote the manuscript. TO supervised the project. All authors contributed to the article and approved the submitted version.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

References

1. Antimicrobial Resistance: Tackling a Crisis for the Health and Wealth of Nations. *The Review on Antimicrobial Resistance, Chaired by Jim O'Neill* (2014). Available at: http://www.jpiamreu/wp-content/uploads/2014/12/AMR-Review-Paper-Tackling-a-crisis-for-the-health-and-wealth-of-nations_1-2.pdf
2. World Health Organization. *Global Tuberculosis Report 2018*. Geneva, Switzerland: World Health Organization (2018). Available at: <http://www.who.int/iris/handle/10665/274453>. License: CC BY-NC-SA 3.0 IGO
3. Waddington CS, Darton TC, Pollard AJ. The Challenge of Enteric Fever. *J infect* (2014) 68 Suppl 1:S38–50. doi: 10.1016/j.jinf.2013.09.013
4. Stanaway JD, Parisi A, Sarkar K, Blacker BF, Reiner RC, Hay SI, et al. The Global Burden of non-Typhoidal Salmonella Invasive Disease: A Systematic Analysis for the Global Burden of Disease Study 2017. *Lancet Infect Dis* (2019) 19(12):1312–24. doi: 10.1016/S1473-3099(19)30418-9
5. Kingsley RA, Msefula CL, Thomson NR, Kariuki S, Holt KE, Gordon MA, et al. Epidemic Multiple Drug Resistant Salmonella Typhimurium Causing Invasive Disease in Sub-Saharan Africa Have a Distinct Genotype. *Genome Res* (2009) 19(12):2279–87. doi: 10.1101/gr.091017.109
6. Mather AE, Phuong TLT, Gao Y, Clare S, Mukhopadhyay S, Goulding DA, et al. New Variant of Multidrug-Resistant *Salmonella enterica* Serovar Typhimurium Associated With Invasive Disease in Immunocompromised Patients in Vietnam. *mBio* (2018) 9(5):e01056–18. doi: 10.1128/mBio.01056-18
7. Palucci I, Delogu G. Host Directed Therapies for Tuberculosis: Futures Strategies for an Ancient Disease. *Chemotherapy* (2018) 63(3):172–80. doi: 10.1159/000490478
8. O'Neill LA, Pearce EJ. Immunometabolism Governs Dendritic Cell and Macrophage Function. *J Exp Med* (2016) 213(1):15–23. doi: 10.1084/jem.20151570
9. Wang T, Liu H, Lian G, Zhang SY, Wang X, Jiang C. HIF1alpha-Induced Glycolysis Metabolism Is Essential to the Activation of Inflammatory Macrophages. *Mediators Inflamm* (2017) 2017:9029327. doi: 10.1155/2017/9029327
10. Gleeson LE, Sheedy FJ, Palsson-McDermott EM, Triglia D, O'Leary SM, O'Sullivan MP, et al. Cutting Edge: *Mycobacterium tuberculosis* Induces Aerobic Glycolysis in Human Alveolar Macrophages That Is Required for Control of Intracellular Bacillary Replication. *J Immunol* (2016) 196(6):2444–9. doi: 10.4049/jimmunol.1501612
11. Palsson-McDermott EM, Curtis AM, Goel G, Lauterbach MA, Sheedy FJ, Gleeson LE, et al. Pyruvate Kinase M2 Regulates Hif-1alpha Activity and IL-1beta Induction and is a Critical Determinant of the Warburg Effect in LPS-Activated Macrophages. *Cell Metab* (2015) 21(1):65–80. doi: 10.1016/j.cmet.2014.12.005
12. Shi L, Salamon H, Eugenin EA, Pine R, Cooper A, Gennaro ML. Infection With *Mycobacterium tuberculosis* Induces the Warburg Effect in Mouse Lungs. *Sci Rep* (2015) 5:18176. doi: 10.1038/srep18176
13. Appelberg R, Moreira D, Barreira-Silva P, Borges M, Silva L, Dinis-Oliveira RJ, et al. The Warburg Effect in Mycobacterial Granulomas Is Dependent on the Recruitment and Activation of Macrophages by Interferon-Gamma. *Immunology* (2015) 145(4):498–507. doi: 10.1111/imm.12464
14. Lachmandas E, Beigier-Bompadre M, Cheng SC, Kumar V, van Laarhoven A, Wang X, et al. Rewiring Cellular Metabolism via the AKT/mTOR Pathway Contributes to Host Defence Against *Mycobacterium tuberculosis* in Human and Murine Cells. *Eur J Immunol* (2016) 46(11):2574–86. doi: 10.1002/eji.201546259
15. Mills EL, Kelly B, O'Neill LAJ. Mitochondria are the Powerhouses of Immunity. *Nat Immunol* (2017) 18(5):488–98. doi: 10.1038/ni.3704
16. Kelly B, O'Neill LA. Metabolic Reprogramming in Macrophages and Dendritic Cells in Innate

- Immunity. *Cell Res* (2015) 25(7):771–84. doi: 10.1038/cr.2015.68
17. Lampropoulou V, Sergushichev A, Bambouskova M, Nair S, Vincent EE, Loginicheva E, et al. Itaconate Links Inhibition of Succinate Dehydrogenase With Macrophage Metabolic Remodeling and Regulation of Inflammation. *Cell Metab* (2016) 24(1):158–66. doi: 10.1016/j.cmet.2016.06.004
 18. Liu PS, Wang H, Li X, Chao T, Teav T, Christen S, et al. Alpha-Ketoglutarate Orchestrates Macrophage Activation Through Metabolic and Epigenetic Reprogramming. *Nat Immunol* (2017) 18(9):985–94. doi: 10.1038/ni.3796
 19. Mills EL, Kelly B, Logan A, Costa ASH, Varma M, Bryant CE, et al. Succinate Dehydrogenase Supports Metabolic Repurposing of Mitochondria to Drive Inflammatory Macrophages. *Cell* (2016) 167(2):457–70.e13. doi: 10.1016/j.cell.2016.08.064
 20. Murphy MP, O'Neill LAJ. Krebs Cycle Reimagined: The Emerging Roles of Succinate and Itaconate as Signal Transducers. *Cell* (2018) 174(4):780–4. doi: 10.1016/j.cell.2018.07.030
 21. Samuvel DJ, Sundararaj KP, Nareika A, Lopes-Virella MF, Huang Y. Lactate Boosts TLR4 Signaling and NF-KappaB Pathway-Mediated Gene Transcription in Macrophages via Monocarboxylate Transporters and MD-2 Up-Regulation. *J Immunol* (2009) 182(4):2476–84. doi: 10.4049/jimmunol.0802059
 22. Tannahill GM, Curtis AM, Adamik J, Palsson-McDermott EM, McGettrick AF, Goel G, et al. Succinate Is an Inflammatory Signal That Induces IL-1beta Through HIF-1alpha. *Nature* (2013) 496(7444):238–42. doi: 10.1038/nature11986
 23. Bekkering S, Arts RJW, Novakovic B, Kourtzelis I, van der Heijden C, Li Y, et al. Metabolic Induction of Trained Immunity Through the Mevalonate Pathway. *Cell* (2018) 172(1-2):135–46.e9. doi: 10.1016/j.cell.2017.11.025
 24. Michelucci A, Cordes T, Ghelfi J, Pailot A, Reiling N, Goldmann O, et al. Immune-Responsive Gene 1 Protein Links Metabolism to Immunity by Catalyzing Itaconic Acid Production. *Proc Natl Acad Sci USA* (2013) 110 (19):7820–5. doi: 10.1073/pnas.1218599110
 25. Verreck FA, de Boer T, Langenberg DM, Hoeve MA, Kramer M, Vaisberg E, et al. Human IL-23-Producing Type 1 Macrophages Promote But IL-10-Producing Type 2 Macrophages Subvert Immunity to (Myco)Bacteria. *Proc Natl Acad Sci USA* (2004) 101(13):4560–5. doi: 10.1073/pnas.0400983101
 26. Verreck FA, de Boer T, Langenberg DM, van der Zanden L, Ottenhoff TH. Phenotypic and Functional Profiling of Human Proinflammatory Type-1 and Anti-Inflammatory Type-2 Macrophages in Response to Microbial Antigens and IFN-Gamma- and CD40L-Mediated Costimulation. *J Leukoc Biol* (2006) 79(2):285–93. doi: 10.1189/jlb.0105015
 27. Rodríguez-Prados J-C, Través PG, Cuenca J, Rico D, Aragonés J, Martín-Sanz P, et al. Substrate Fate in Activated Macrophages: A Comparison Between Innate, Classic, and Alternative Activation. *J Immunol* (2010) 185(1):605–14. doi: 10.4049/jimmunol.0901698
 28. Jha AK, Huang SC, Sergushichev A, Lampropoulou V, Ivanova Y, Loginicheva E, et al. Network Integration of Parallel Metabolic and Transcriptional Data Reveals Metabolic Modules That Regulate Macrophage Polarization. *Immunity* (2015) 42(3):419–30. doi: 10.1016/j.immuni.2015.02.005
 29. Galvan-Pena S, O'Neill LA. Metabolic Reprograming in Macrophage Polarization. *J Immunol* (2014) 192(5):420. doi: 10.1093/imm.192.5.420
 30. Patel MS, Korotchkina LG. Regulation of Mammalian Pyruvate Dehydrogenase Complex by Phosphorylation: Complexity of Multiple Phosphorylation Sites and Kinases. *Exp Mol Med* (2001) 33(4):191–7. doi: 10.1038/emmm.2001.32
 31. Harris RA, Bowker-Kinley MM, Huang B, Wu P. Regulation of the Activity of the Pyruvate Dehydrogenase Complex. *Adv Enzyme Regul* (2002) 42:249–59. doi: 10.1016/S0065-2571(01)00061-9
 32. Bonnet S, Archer SL, Allalunis-Turner J, Haromy A, Beaulieu C, Thompson R, et al. A Mitochondria-

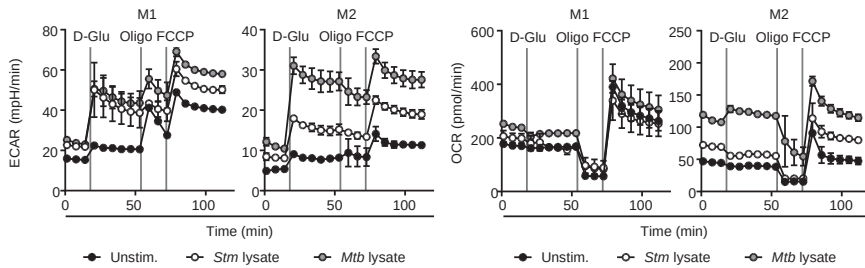
- K⁺ Channel Axis Is Suppressed in Cancer and its Normalization Promotes Apoptosis and Inhibits Cancer Growth. *Cancer Cell* (2007) 11(1):37–51. doi: 10.1016/j.ccr.2006.10.020
33. Mayers RM, Butlin RJ, Kilgour E, Leighton B, Martin D, Myatt J, et al. AZD7545, a Novel Inhibitor of Pyruvate Dehydrogenase Kinase 2 (PDHK2), Activates Pyruvate Dehydrogenase *In Vivo* and Improves Blood Glucose Control in Obese (Fa/Fa) Zucker Rats. *Biochem Soc Trans* (2003) 31(Pt 6):1165–7. doi: 10.1042/bst0311165
 34. Stacpoole PW, Moore GW, Kornhauser DM. Metabolic Effects of Dichloroacetate in Patients With Diabetes Mellitus and Hyperlipoproteinemia. *N Engl J Med* (1978) 298(10):526–30. doi: 10.1056/NEJM197803092981002
 35. Ty MC, Pn L, Alberola J, Rodriguez A, Rodriguez-Cortes A. Immuno-Metabolic Profile of Human Macrophages After Leishmania and Trypanosoma Cruzi Infection. *PLoS One* (2019) 14(12):e0225588. doi: 10.1371/journal.pone.0225588
 36. Vijayan V, Pradhan P, Braud L, Fuchs HR, Gueler F, Motterlini R, et al. Human and Murine Macrophages Exhibit Differential Metabolic Responses to Lipopolysaccharide—a Divergent Role for Glycolysis. *Redox Biol* (2019) 22:101147. doi: 10.1016/j.redox.2019.101147
 37. Hackett EE, Charles-Messance H, O’Leary SM, Gleeson LE, Munoz-Wolf N, Case S, et al. *Mycobacterium tuberculosis* Limits Host Glycolysis and IL-1 β by Restriction of PFK-M via MicroRNA-21. *Cell Rep* (2020) 30(1):124–36.e4. doi: 10.1016/j.celrep.2019.12.015
 38. Cumming BM, Addicott KW, Adamson JH, Steyn AJ. *Mycobacterium tuberculosis* Induces Decelerated Bioenergetic Metabolism in Human Macrophages. *eLife* (2018) 7:e39169. doi: 10.7554/eLife.39169
 39. Braverman J, Sogi KM, Benjamin D, Nomura DK, Stanley SA. HIF-1 α Is an Essential Mediator of IFN- γ -Dependent Immunity to *Mycobacterium tuberculosis*. *J Immunol* (2016) 197(4):1287–97. doi: 10.4049/jimmunol.1600266
 40. Huang L, Nazarova EV, Tan S, Liu Y, Russell DG. Growth of *Mycobacterium tuberculosis* *In Vivo* Segregates With Host Macrophage Metabolism and Ontogeny. *J Exp Med* (2018) 215(4):1135–52. doi: 10.1084/jem.20172020
 41. Kuijl C, Savage ND, Marsman M, Tuin AW, Janssen L, Egan DA, et al. Intracellular Bacterial Growth is Controlled by a Kinase Network Around PKB/Akt1. *Nature* (2007) 450(7170):725–30. doi: 10.1038/nature06345
 42. Korbee CJ, Heemskerk MT, Kocev D, van Strijen E, Rabiee O, Franken K, et al. Combined Chemical Genetics and Data-Driven Bioinformatics Approach Identifies Receptor Tyrosine Kinase Inhibitors as Host-Directed Antimicrobials. *Nat Commun* (2018) 9(1):358. doi: 10.1038/s41467-017-02777-6
 43. Price JV, Jiang K, Galantowicz A, Freifeld A, Vance RE. *Legionella pneumophila* Is Directly Sensitive to 2-Deoxyglucose-Phosphate via Its UhpC Transporter But Is Indifferent to Shifts in Host Cell Glycolytic Metabolism. *J Bacteriol* (2018) 200(16):e00176–18. doi: 10.1128/JB.00176-18
 44. Wang F, Zhang S, Vuckovic I, Jeon R, Lerman A, Folmes CD, et al. Glycolytic Stimulation Is Not a Requirement for M2 Macrophage Differentiation. *Cell Metab* (2018) 28(3):463–75.e4. doi: 10.1016/j.cmet.2018.08.012
 45. Blischak JD, Tailleux L, Mitrano A, Barreiro LB, Gilad Y. *Mycobacterial* Infection Induces a Specific Human Innate Immune Response. *Sci Rep* (2015) 5:16882. doi: 10.1038/srep16882
 46. Sanchez WY, McGee SL, Connor T, Mottram B, Wilkinson A, Whitehead JP, et al. Dichloroacetate Inhibits Aerobic Glycolysis in Multiple Myeloma Cells and Increases Sensitivity to Bortezomib. *Br J Cancer* (2013) 108(8):1624–33. doi: 10.1038/bjc.2013.120
 47. Rardin MJ, Wiley SE, Naviaux RK, Murphy AN, Dixon JE. Monitoring Phosphorylation of the Pyruvate Dehydrogenase Complex. *Anal Biochem* (2009) 389(2):157–64. doi: 10.1016/j.ab.2009.03.040
 48. Shen H, Hau E, Joshi S, Dilda PJ, McDonald KL. Sensitization of Glioblastoma Cells to Irradiation

- by Modulating the Glucose Metabolism. *Mol Cancer Ther* (2015) 14(8):1794–804. doi: 10.1158/1535-7163.MCT-15-0247
49. De Preter G, Neveu MA, Danhier P, Brisson L, Payen VL, Porporato PE, et al. Inhibition of the Pentose Phosphate Pathway by Dichloroacetate Unravels a Missing Link Between Aerobic Glycolysis and Cancer Cell Proliferation. *Oncotarget* (2016) 7(3):2910–20. doi: 10.18632/oncotarget.6272
 50. Kluza J, Corazao-Rozas P, Touil Y, Jendoubi M, Maire C, Guerreschi P, et al. Inactivation of the HIF-1 α /PDK3 Signaling Axis Drives Melanoma Toward Mitochondrial Oxidative Metabolism and Potentiates the Therapeutic Activity of Pro-Oxidants. *Cancer Res* (2012) 72(19):5035–47. doi: 10.1158/0008-5472.CAN-12-0979
 51. Selak MA, Armour SM, MacKenzie ED, Boulahbel H, Watson DG, Mansfield KD, et al. Succinate Links TCA Cycle Dysfunction to Oncogenesis by Inhibiting HIF- α Prolyl Hydroxylase. *Cancer Cell* (2005) 7(1):77–85. doi: 10.1016/j.ccr.2004.11.022
 52. Florio R, De Lellis L, Veschi S, Verginelli F, di Giacomo V, Gallorini M, et al. Effects of Dichloroacetate as Single Agent or in Combination With GW6471 and Metformin in Paraganglioma Cells. *Sci Rep* (2018) 8(1):1–14. doi: 10.1038/s41598-018-31797-5
 53. McCall CE, Zabalawi M, Liu T, Martin A, Long DL, Buechler NL, et al. Pyruvate Dehydrogenase Complex Stimulation Promotes Immunometabolic Homeostasis and Sepsis Survival. *JCI Insight* (2018) 3(15):e99292. doi: 10.1172/jci.insight.99292
 54. Van den Bossche J, Baardman J, de Winther MP. Metabolic Characterization of Polarized M1 and M2 Bone Marrow-Derived Macrophages Using Real- Time Extracellular Flux Analysis. *J Vis Exp* (2015) 105:e53424. doi: 10.3791/53424
 55. Chiba S, Hisamatsu T, Suzuki H, Mori K, Kitazume MT, Shimamura K, et al. Glycolysis Regulates LPS-Induced Cytokine Production in M2 Polarized Human Macrophages. *Immunol Lett* (2017) 183:17–23. doi: 10.1016/j.imlet.2017.01.012
 56. Arts RJ, Carvalho A, La Rocca C, Palma C, Rodrigues F, Silvestre R, et al. Immunometabolic Pathways in BCG-Induced Trained Immunity. *Cell Rep* (2016) 17(10):2562–71. doi: 10.1016/j.celrep.2016.11.011
 57. Kurtoglu M, Gao N, Shang J, Maher JC, Lehrman MA, Wangpaichitr M, et al. Under Normoxia, 2-Deoxy-D-Glucose Elicits Cell Death in Select Tumor Types Not by Inhibition of Glycolysis But by Interfering With N-Linked Glycosylation. *Mol Cancer Ther* (2007) 6(11):3049–58. doi: 10.1158/1535-7163.MCT-07-0310
 58. Ralser M, Wamelink MM, Struys EA, Joppich C, Krobitsch S, Jakobs C, et al. A Catabolic Block Does Not Sufficiently Explain How 2-Deoxy-D-Glucose Inhibits Cell Growth. *Proc Natl Acad Sci USA* (2008) 105(46):17807–11. doi: 10.1073/pnas.0803090105
 59. James MO, Jahn SC, Zhong G, Smeltz MG, Hu Z, Stacpoole PW. Therapeutic Applications of Dichloroacetate and the Role of Glutathione Transferase Zeta- 1. *Pharmacol Ther* (2017) 170:166–80. doi: 10.1016/j.pharmthera.2016.10.018
 60. Tataranni T, Piccoli C. Dichloroacetate (DCA) and Cancer: An Overview Towards Clinical Applications. *Oxid Med Cell Longev* (2019) 2019:8201079. doi: 10.1155/2019/8201079
 61. Abdelmalak M, Lew A, Ramezani R, Shroads AL, Coats BS, Langae T, et al. Long-Term Safety of Dichloroacetate in Congenital Lactic Acidosis. *J Mol Genet Metab* (2013) 109(2):139–43. doi: 10.1016/j.ymgme.2013.03.019
 62. Chu QS, Sangha R, Spratlin J, Vos LJ, Mackey JR, McEwan AJ, et al. A Phase I Open-Labeled, Single-Arm, Dose-Escalation, Study of Dichloroacetate (DCA) in Patients With Advanced Solid Tumors. *Invest New Drugs* (2015) 33(3):603–10. doi: 10.1007/s10637-015-0221-y
 63. Kaufmann P, Engelstad K, Wei Y, Jhung S, Sano M, Shungu D, et al. Dichloroacetate Causes Toxic Neuropathy in MELAS: A Randomized, Controlled Clinical Trial. *J Neurol* (2006) 253(3):324–30. doi: 10.1007/s00415-006-0591-3
 64. Stacpoole PW, Martyniuk CJ, James MO, Calcutt NA. Dichloroacetate- Induced Peripheral

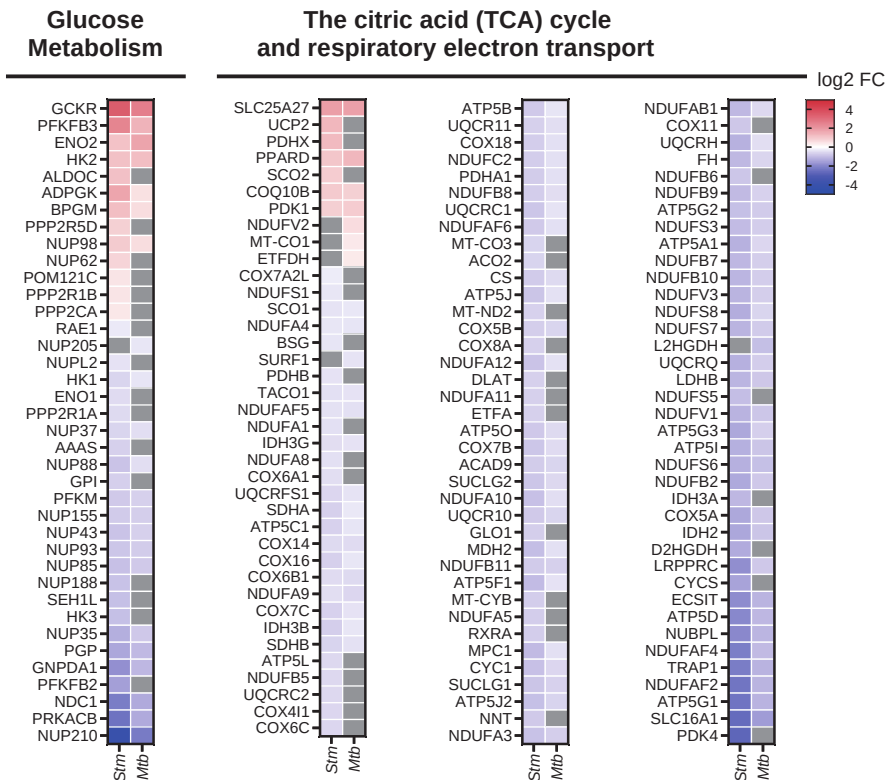
- Neuropathy. *J Int Rev Neurobiol* (2019) 145:211–38. doi: 10.1016/bs.irn.2019.05.003
65. Stacpoole PW, Martyniuk CJ, James MO, Calcutt NA. Chapter Eight - Dichloroacetate-Induced Peripheral Neuropathy. In: P Fernyhough, NA Calcutt, editors. *International Review of Neurobiology*, vol. 145. Oxford, United Kingdom: Academic Press (2019). p. 211–38. doi: 10.1016/bs.irn.2019.05.003
 66. Majowicz SE, Musto J, Scallan E, Angulo FJ, Kirk M, O'Brien SJ, et al. The Global Burden of Nontyphoidal Salmonella Gastroenteritis. *Clin Infect Dis* (2010) 50(6):882–9. doi: 10.1086/650733
 67. Marzel A, Desai PT, Goren A, Schorr YI, Nissan I, Porwollik S, et al. Persistent Infections by Nontyphoidal Salmonella in Humans: Epidemiology and Genetics. *J Clin Infect Dis* (2016) 62(7):879–86. doi: 10.1093/cid/civ1221
 68. Rodrigues AS, Correia M, Gomes A, Pereira SL, Perestrelo T, Sousa MI, et al. Dichloroacetate, the Pyruvate Dehydrogenase Complex and the Modulation of mESC Pluripotency. *PloS One* (2015) 10(7):e0131663. doi: 10.1371/journal.pone.0131663
 69. Rosenberg G, Yehezkel D, Hoffman D, Mattioli CC, Fremder M, Ben-Arosh H, et al. Host Succinate is an Activation Signal for Salmonella Virulence During Intracellular Infection. *Science* (2021) 371(6527):400–5. doi: 10.1126/science.aba8026
 70. Gillis CC, Hughes ER, Spiga L, Winter MG, Zhu W, de Carvalho TF, et al. Dysbiosis-Associated Change in Host Metabolism Generates Lactate to Support Salmonella Growth. *Cell Host Microbe* (2018) 23(1):54–64. doi: 10.1016/j.chom.2017.11.006
 71. Jha MK, Song GJ, Lee MG, Jeoung NH, Go Y, Harris RA, et al. Metabolic Connection of Inflammatory Pain: Pivotal Role of a Pyruvate Dehydrogenase Kinase-Pyruvate Dehydrogenase-Lactic Acid Axis. *J Neurosci* (2015) 35 (42):14353–69. doi: 10.1523/JNEUROSCI.1910-15.2015
 72. Na YR, Jung D, Song J, Park JW, Seok SH. Pyruvate Dehydrogenase Kinase is a Negative Regulator of Interleukin-10 Production in Macrophages. *J Mol Cell Biol* (2020) 12(7):543–55. doi: 10.1093/jmcb/mjz113
 73. Zhu X, Long D, Zabalawi M, Ingram B, Yoza BK, Stacpoole PW, et al. Stimulating Pyruvate Dehydrogenase Complex Reduces Itaconate Levels and Enhances TCA Cycle Anabolic Bioenergetics in Acutely Inflamed Monocytes. *J Leukoc Biol* (2020) 107(3):467–84. doi: 10.1002/JLB.3A1119-236R
 74. Roh J-L, Park JY, Kim EH, Jang HJ, Kwon M. Activation of Mitochondrial Oxidation by PDK2 Inhibition Reverses Cisplatin Resistance in Head and Neck Cancer. *Cancer Lett* (2016) 371(1):20–9. doi: 10.1016/j.canlet.2015.11.023
 75. Mastroeni P, Vazquez-Torres A, Fang FC, Xu Y, Khan S, Hormaeche CE, et al. Antimicrobial Actions of the NADPH Phagocyte Oxidase and Inducible Nitric Oxide Synthase in Experimental Salmonellosis. II. Effects on Microbial Proliferation and Host Survival *In Vivo*. *J Exp Med* (2000) 192 (2):237–48. doi: 10.1084/jem.192.2.237
 76. Shiloh MU, MacMicking JD, Nicholson S, Brause JE, Potter S, Marino M, et al. Phenotype of Mice and Macrophages Deficient in Both Phagocyte Oxidase and Inducible Nitric Oxide Synthase. *Immunity* (1999) 10(1):29–38. doi: 10.1016/S1074-7613(00)80004-7
 77. West AP, Brodsky IE, Rahner C, Woo DK, Erdjument-Bromage H, Tempst P, et al. TLR Signalling Augments Macrophage Bactericidal Activity Through Mitochondrial ROS. *Nature* (2011) 472(7344):476–80. doi: 10.1038/nature09973
 78. Rhen M. Salmonella and Reactive Oxygen Species: A Love-Hate Relationship. *J Innate Immun* (2019) 11(3):216–26. doi: 10.1159/000496370
 79. Van der Ven BC, Yates RM, Russell DG. Intraphagosomal Measurement of the Magnitude and Duration of the Oxidative Burst. *Traffic (Copenhagen Denmark)* (2009) 10(4):372–8. doi: 10.1111/j.1600-0854.2008.00877.x
 80. Vazquez-Torres A, Fang FC. Salmonella Evasion of the NADPH Phagocyte Oxidase. *Microbes*

- Infect* (2001) 3(14-15):1313–20. doi: 10.1016/S1286-4579 (01)01492-7
81. Moreira JD, Koch BEV, van Veen S, Walburg KV, Vrieling F, Mara Pinto Dabés Guimarães T, et al. Functional Inhibition of Host Histone Deacetylases (HDACs) Enhances *In Vitro* and *In Vivo* Anti-Mycobacterial Activity in Human Macrophages and in Zebrafish. *Front Immunol* (2020) 11(36):36. doi: 10.3389/fimmu.2020.00036
 82. Vrieling F, Kostidis S, Spaink HP, Haks MC, Mayboroda OA, Ottenhoff TH, et al. Analyzing the Impact of *Mycobacterium tuberculosis* Infection on Primary Human Macrophages by Combined Exploratory and Targeted Metabolomics. *Sci Rep* (2020) 10(1):1–13. doi: 10.1038/s41598-020-62911-1
 83. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, et al. Fiji: An Open-Source Platform for Biological-Image Analysis. *Nat Methods* (2012) 9(7):676–82. doi: 10.1038/nmeth.2019

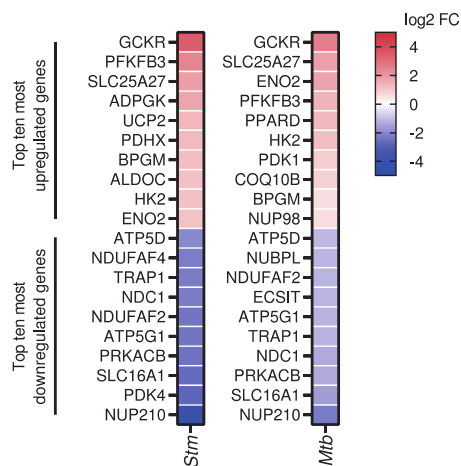
Supplementary Figures



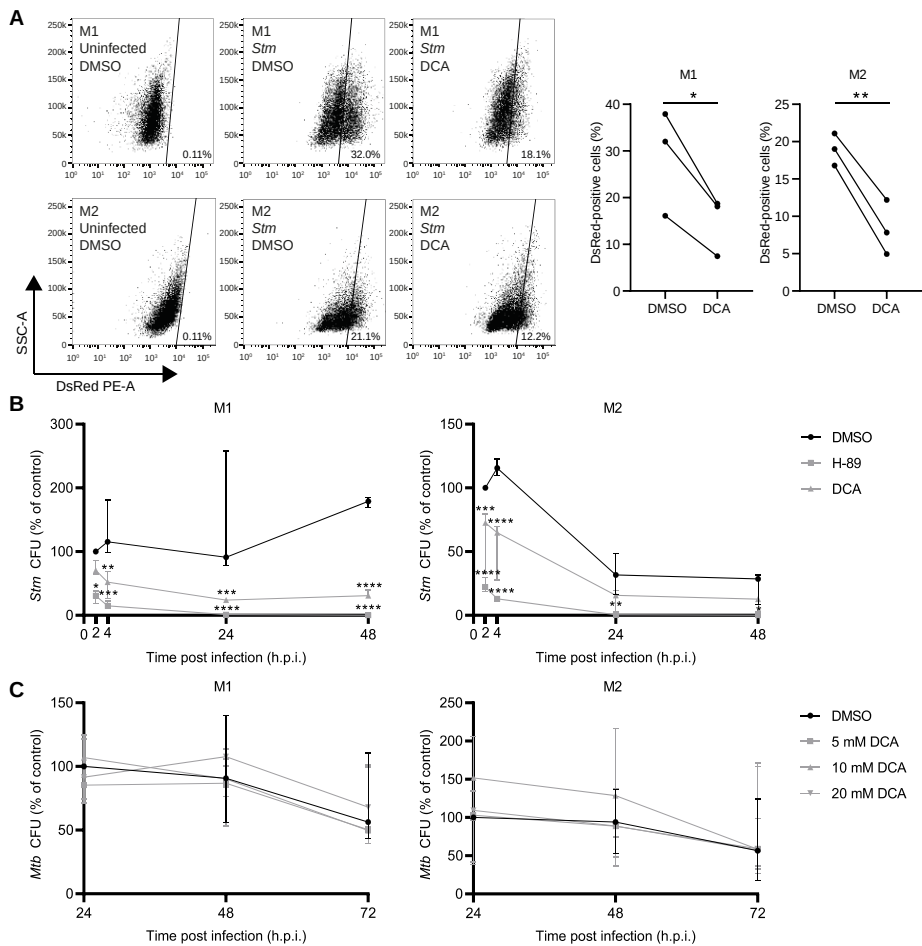
SUPPLEMENTAL FIGURE 1 | *Stm* and *Mtb* induced a more glycolytic phenotype in human macrophages compared to uninfected cells. M1 and M2 were unstimulated (unstim.) or stimulated with 10 $\mu\text{g}/\text{ml}$ *Stm* or *Mtb* lysate for 4 hours and inserted in the Seahorse analyzer. D-Glucose (10 mM), oligomycin (oligo, 1 μM) and carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP, 2 μM) were sequentially injected after 18, 54 and 72 minutes, respectively. ECAR and OCR profiles of one representative donor out of four donors analyzed. Data represent the mean $\pm$ S.D. of triplicates.



SUPPLEMENTAL FIGURE 2 | Metabolic genes were regulated in human macrophages upon *Stm* and *Mtb* infection. Heatmap displaying median log2 fold changes (FC) of all genes involved in 'glucose metabolism' and 'the citric acid (TCA) cycle and respiratory electron transport' in M2 obtained from six donors 18 hours post *Stm* or *Mtb* infection (MOI 5) compared to uninfected controls. Genes that were significantly up- or downregulated by limma-voom (adjusted p-value < 0.05) are shown using a red to white to blue color scale. Grey indicates non-significant genes. Data obtained from Blischak et al. (45).



SUPPLEMENTAL FIGURE 3 | Top ten most upregulated and top ten most downregulated metabolic genes in human macrophages upon *Stm* and *Mtb* infection. Heatmap displaying median log2 fold changes (FC) of the top ten most upregulated or downregulated genes involved in 'glucose metabolism' and 'the citric acid (TCA) cycle and respiratory electron transport' in M2 obtained from six donors 18 hours post *Stm* or *Mtb* infection (MOI 5) compared to uninfected controls. Genes that were significantly up- or downregulated by limma-voom (adjusted p-value < 0.05) are shown using a red to white to blue color scale. Data obtained from Blischak et al. (45).



SUPPLEMENTAL FIGURE 4 | DCA markedly inhibited bacterial outgrowth of *Stm* but not *Mtb*. (A)

Flow cytometer plots of *Stm*-infected M1 and M2 that were treated with 10 mM DCA or vehicle control DMSO (0.1% v/v) overnight. Shown are the side scatter area on the y-axis and the DsRed-PE area on the x-axis of one representative donor (left panel) and the quantified bacterial load (DsRed-positive cells) as percentage of the total cell number (right panel). Lines connect data points from the same donor, with a total of three donors tested. Cells were gated for DsRed-positivity based on uninfected control cells. Differences were significant by a paired t-test. (B) *Stm* CFUs following treatment with H-89 (10 μ M), DCA (10 mM) or vehicle control DMSO (0.1% v/v) at 2, 4, 24 or 48 h.p.i. Data represent the median $\pm$ range of three donors. CFUs are expressed as percentage of DMSO. Differences were significant by RM Two-way ANOVA with Dunnett's-multiple comparison test against DMSO. (C) *Mtb* CFUs following treatment with DCA (5-20 mM) or vehicle control DMSO (0.1% v/v) at 24, 48 or 72 h.p.i. Data represent the median $\pm$ range of four donors. CFUs are expressed as percentage of DMSO. Differences were tested by RM Two-way ANOVA with Dunnett's-multiple comparison test against DMSO. Dots represent the mean of triplicates from a single donor. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

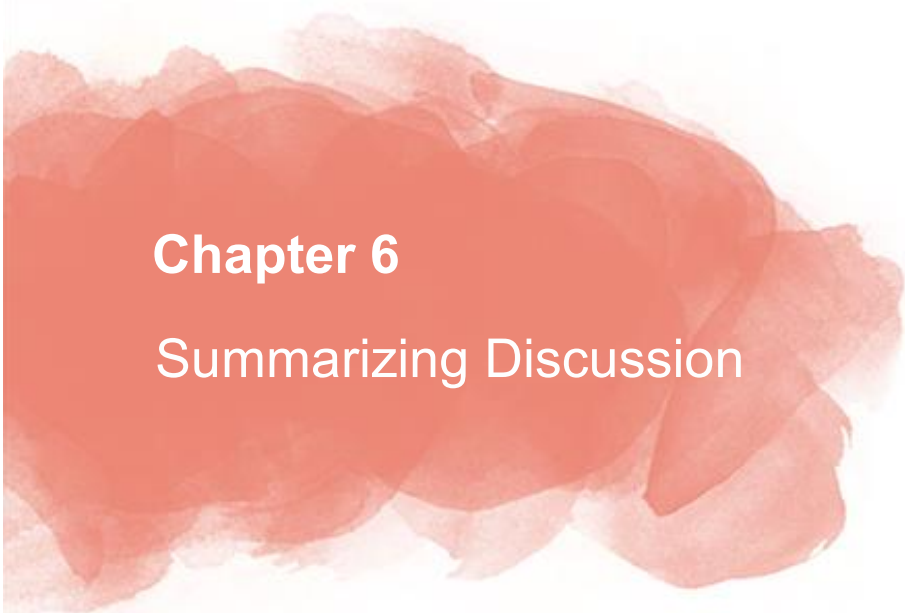
Supplementary Tables

Supplemental Table 1 | *Stm* infection induces cytokine/chemokine production in human macrophages and DCA has little effect on cytokine/chemokine production on top of infection.

		M1			M2		
		Mock	<i>Stm</i>	<i>Mtb</i>	Mock	<i>Stm</i>	<i>Mtb</i>
Growth factors	EGF	10	18	16	9	26	19
	FGF-2	75	98	71	54	81	67
	Ftl-3L	24	34	31	20	36	28
	PDGF-AB/BB	18	45	24	24	41	43
	VEGF	89	155	91	49	127	91
Anti-inflammatory cytokines	TGF α	12	24	11	2	23	8
	IL-10	7	68	7	9	608	327
	IL-13	6	9	7	2	9	5
	IL-1Ra	2967	5282	2799	388	2956	554
	IL-4	29	151	47	51	229	173
Pro-inflammatory cytokines	G-CSF	153	1171	185	120	3804	359
	GM-CSF	20	433	32	9	250	37
	IFN- α 2	53	96	68	42	83	63
	IFN- γ	9	22	12	3	29	6
	IL-12p40	34	372	49	21	57	35
	IL-12p70	4	16	4	5	30	5
	IL-15	4	6	3	5	12	5
	sCD40L	15	32	7	18	39	17
	IL17A	5	9	6	6	6	7
	IL-1 α	5	14	7	9	38	12
	IL-9	8	14	10	8	14	10
	IL-1 β	5	285	7	4	1168	67
	IL-6	6	1908	17	3	4970	129
	IL-7	15	105	21	17	137	36
	IL-8	590	12676	2363	9451	12767	13563
	TNF α	32	10000	135	24	10000	593
Chemokines	MDC	29426	28171	27763	601	320	466
	Eotaxin	14	28	19	17	26	24
	MCP-1	1065	8902	1240	1250	9472	4427
	MIP-1 α	254	12104	944	96	11151	3513
	MIP-1 β	288	30991	772	98	30225	2119
	RANTES	19	1789	57	67	3921	252
	MCP-3	277	4187	331	57	711	138
	Fractalkine	190	401	193	224	489	312
	GRO	72	1076446	606	1349	2145300	251082
	CXCL10	47	384	81	828	2781	575

		M1				M2			
		<i>Stm</i>		<i>Mtb</i>		<i>Stm</i>		<i>Mtb</i>	
		DMSO	DCA	DMSO	DCA	DMSO	DCA	DMSO	DCA
Growth factors	EGF	18	20	16	14	26	19	19	17
	FGF-2	98	93	71	58	81	83	67	67
	Ftl-3L	34	43	31	24	36	28	28	14
	PDGF-AB/BB	45	33	24	29	41	34	43	18
	VEGF	155	126	91	78	127	125	91	114
Anti-inflammatory cytokines	TGF α	24	15	11	9	23	14	8	8
	IL-10	68	12	7	7	608	609	327	128
	IL-13	9	11	7	6	9	11	5	7
	IL-1Ra	5282	4865	2799	2861	2956	2288	554	538
	IL-4	151	155	47	51	229	233	173	224
Pro-inflammatory cytokines	G-CSF	1171	1179	185	216	3804	5275	359	562
	GM-CSF	433	500	32	28	250	411	37	64
	IFN- α 2	96	108	68	70	83	94	63	66
	IFN- γ	22	26	12	10	29	22	6	5
	IL-12p40	372	487	49	35	57	76	35	35
	IL-12p70	16	25	4	5	30	30	5	5
	IL-15	6	7	3	4	12	11	5	6
	sCD40L	32	32	7	22	39	34	17	15
	IL17A	9	10	6	8	6	7	7	6
	IL-1 α	14	20	7	6	38	37	12	15
	IL-9	14	17	10	10	14	14	10	9
	IL-1 β	285	704	7	6	1168	1249	67	145
	IL-6	1908	1876	17	17	4970	5007	129	386
	IL-7	105	92	21	20	137	140	36	51
	IL-8	12676	13176	2363	3580	12767	13523	13563	13466
	TNF α	10000	10000	135	210	10000	10000	593	1526
Chemokines	MDC	28171	38029	27763	58604	320	434	466	1112
	Eotaxin	28	29	19	22	26	29	24	25
	MCP-1	8902	7437	1240	961	9472	7591	4427	4365
	MIP-1 α	12104	11242	944	1368	11151	10334	3513	9063
	MIP-1 β	30991	15365	772	795	30225	24640	2119	2834
	RANTES	1789	1876	57	72	3921	5150	252	448
	MCP-3	4187	2270	331	335	711	625	138	232
	Fractalkine	401	480	193	248	489	433	312	290
	GRO	1076446	76066	606	606	2145300	2145300	251082	2145300
	CXCL10	384	139	81	96	2781	2385	575	732

Shown are median cytokine/chemokine levels (in pg/ml) of 6 different donors in supernatants of M1 and M2 following *Stm*, *Mtb* or mock infection overnight or in supernatants of *Stm*- or *Mtb*-infected M1 and M2 exposed to DCA (10 mM) or an equal volume of DMSO (0.1% v/v) overnight. Of the 41 analytes measured, only cytokines/chemokines that were above 10 pg/ml in at least one experimental condition are shown.

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Chapter 6

Summarizing Discussion

Cassandra L.R. van Doorn

Introduction

Bacterial virulence, host genetic and host non-genetic immune factors underly and regulate tuberculosis (TB) pathogenesis. A rapid and successful immune response is required to eliminate *Mtb*, but on the other hand disease-associated immune responses characterize, and can be used as correlates of, TB disease activity. In addition, host immunity can be targeted by compounds (host-directed therapy, HDT) to improve mycobacterial control. The challenges of developing better diagnostic tools, vaccines and therapies for TB are formidable despite significant progress in each of these fields (1, 2). Comorbidities like HIV and type 2 diabetes (DM) significantly affect TB disease susceptibility and severity. TB-DM comorbidity complicates TB diagnosis and treatment, as indicated by the lower diagnostic accuracy of active TB in patients with concomitant DM (3) and by the impaired treatment success in TB-DM patients compared to TB-only patients (4), respectively. The studies described in this thesis aimed to identify TB-DM associated host-biomarkers as well as to discover and evaluate novel drug candidates for HDT that target intracellular pathogens such as *Mtb* as well as *Salmonella* bacteria, another leading human pathogen. First, we used transcriptomic-based biomarkers in TB-DM patients and patients with TB-related hyperglycaemia, and described the potential of such TB-DM biomarkers in TB-DM-endemic areas. Second, we conducted *in vitro* studies using primary human macrophages to identify potential drug candidates and host targets for the development of HDT. In this final chapter, the findings described in this thesis and potential implications for future research will be discussed.

Improving TB diagnostics: identification of host immune biomarkers

TB appears to be the main driver of transcriptomic differences between TB-DM patients and healthy controls (3, 5). Nevertheless, the TB transcriptome is modulated by concurrent DM, in that TB-DM patients display increased inflammatory and decreased adaptive immune responses (T-cell activation, differentiation, and B cells) compared to patients with uncomplicated TB (3). Before our work, it was unknown whether TB-DM patients also might display a kinetically different transcriptomic response throughout anti-TB treatment compared to TB patients without DM.

In **Chapter 2 and 3**, we describe the transcriptomic response to anti-TB treatment in patients with uncomplicated TB or with TB and DM-comorbidity. TB patients with concomitant hyperglycaemia or DM displayed increased gene expression perturbation at diagnosis compared to TB-only patients, which remained throughout anti-TB treatment (**Chapter 2**). In contrast with TB-only patients, TB-DM patients exhibited increased

expression of T cell subset markers (*e.g.*, *CD8A*, *PTPRCv1*, *CD3E*) and Th2 associated genes (*e.g.*, *GATA3*, *IL-13*) throughout anti-TB treatment. This was caused by lower expression of the aforementioned genes at diagnosis in TB-DM patients versus TB-only patients, and expression of these genes normalized towards TB-only patients throughout anti-TB treatment. Considering these genes, TB-only patients were more similar to healthy controls compared to TB-DM patients. This is particularly interesting because DM patients did not exhibit lower expression of these genes versus healthy controls, in our South African cohort and in a published USA cohort (6), and thus transcriptomic interference by DM does not explain the lower expression of these genes at diagnosis in TB-DM patients. Interestingly, some of these genes were associated with treatment outcome, and three genes (*i.e.*, *CD3E*, *PTPRCv1*, and *GATA3*) were even part of our treatment outcome biomarker signature (**Chapter 3**). Low *CD3E* expression at diagnosis was associated with a poor versus a good treatment outcome, and *CD3E* expression was increased throughout anti-TB treatment in both patient groups. *PTPRCv1* expression was similar between patients with a poor versus a good treatment outcome at diagnosis, but was more increased throughout anti-TB treatment in the poor treatment outcome group. *GATA3* and *IL-13* expression levels were lower in the patients with a poor versus good treatment outcome, and remained lower throughout anti-TB treatment. Interestingly, *IL-13* expression was, together with *AIRE* expression, part of a biomarker signature for TB progression, and *IL-13* expression levels were higher in TB-HIV patients that progressed to TB compared to HIV-patients that did not develop TB (7). Together, our results suggest that genes with differential longitudinal expression in TB-DM versus TB-only patients also showed different kinetics in response to anti-TB treatment when stratified by treatment outcome. The correlation between TB-DM-associated genes and treatment outcome was likely not caused by a bias by DM in the treatment outcome analysis, since the proportion of TB-DM patients was only slightly higher in the poor versus good treatment outcome group (38% versus 32%).

Despite differences in the transcriptomic response to anti-TB treatment between TB-only and TB-DM patients, these patients displayed qualitative and kinetic similarities regarding the regulation of TB-associated key genes (**Chapter 2**). The blood transcriptome of TB patients is characterized by a neutrophil-driven interferon (IFN) response (8-11), and the expression of IFN-inducible genes was downregulated through anti-TB treatment in TB-only patients as well as TB-DM patients. In addition, levels of *GNLY* and *MMP9* - genes that are associated with active TB (12, 13) - normalized throughout anti-TB treatment in TB-DM patients.

The presence of an anti-TB treatment response in TB-DM patients motivated us to identify a universal gene signature for TB treatment response, that can be applied

irrespective of glycemic index (**Chapter 2**). Such signatures can contribute to the development of TB diagnostic assays in TB-DM-endemic areas. We identified a 15-gene signature characterizing the TB treatment response with high classification power between gene expression at diagnosis versus six months of anti-TB treatment (AUCs = 0.88 for TB-only, AUC = 0.85 for TB-DM). Almost all genes in our 15-gene signature (except *CCL13*, *TNF*, and *ZNF532*) appear in multiple published gene signatures for active TB (8, 13-18), TB treatment (14, 15, 19-21) and TB progression (22) (Figure 1). As expected, many genes among the 15-gene signature are involved in IFN signaling (*e.g.*, *FCGR1A*, *GBP5*, *IFIT5*, *TAP1*). In contrast with the majority of IFN-signaling genes, which are downregulated throughout anti-TB treatment, *IFIT5* was significantly upregulated during anti-TB treatment in patients with hyperglycaemia, pre-DM or DM, but not in patients with TB-only. This was particularly unexpected because *IFIT5* was previously associated with active TB (8), and this gene was expected to be downregulated upon anti-TB treatment. Why *IFIT5* fails to be downregulated during anti-TB treatment in DM patients and how this is related to TB and/or DM pathogenesis remains to be investigated. Remaining genes in our gene signature are associated with specific immune cell subsets: B cells (*i.e.*, *CD19*), T helper 1 cells (*i.e.*, *TNF*), regulatory T cells (*i.e.*, *CCL4*), T cell subsets (*i.e.*, *CD3E*, *CD4*, *PTPRCv1*), and myeloid cells (*i.e.*, *FPR1*).

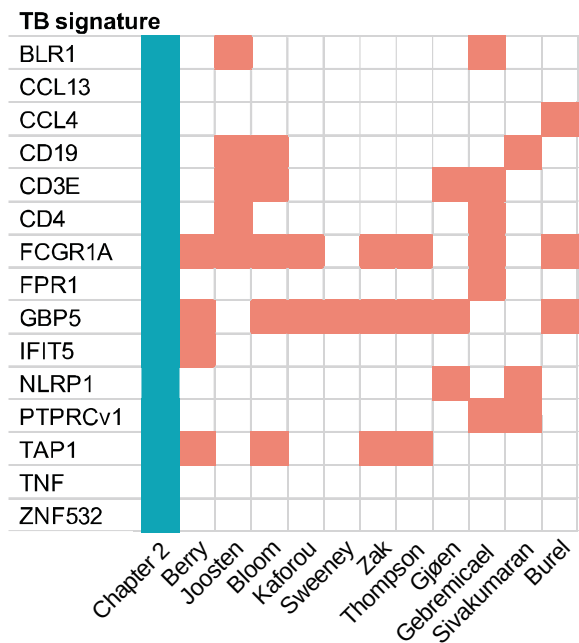


FIGURE 1 | TB treatment response signature in comparison with published TB signatures.

To our knowledge, three genes (*i.e.*, *CCL13*, *TNF*, and *ZNF532*) of our treatment response signature were, although associated with TB, not part of previously published TB signatures. *CCL13* (C-C Motif Chemokine Ligand 3) is a chemokine that is mainly responsible for macrophage and lymphocyte homing to tissues and is involved in granuloma formation (23), and is increased in the bronchoalveolar lavage fluid of TB patients (24). The appearance of *TNF* (tumor necrosis factor) in our TB treatment response signature might result from the inclusion of DM patients in our cohort. It has been reported that *TNF- α* was increased in TB-DM patients compared to patients with uncomplicated TB (25, 26). Moreover, *TNF- α* is secreted by adipocytes in obese and diabetic mice, and *TNF- α* was directly linked with insulin-resistance in mice (27). Finally, *TNF- α* plays a role in granuloma formation and macrophage activation, and asymptomatic TB-infected patients on anti-*TNF* therapy have a higher risk of developing TB if not receiving preventive TB treatment (28). Little is known about *ZNF532* (zinc finger protein 532), apart from its potential transcriptional regulation of adipogenesis (29). *ZNF532* expression was marginally upregulated among statin users who developed type 2 DM (30), and thus the high relative number of DM patients in our cohort might explain the appearance of this gene in our treatment response signature.

In **Chapter 3**, we aimed to identify gene signatures that *predicted* anti-TB treatment outcome in TB and TB-DM patients. Our data showed that patients with a poor treatment outcome displayed a transcriptomic response to anti-TB treatment, both at the level of individual key genes associated with active TB and of global gene expression (*i.e.*, gene expression was perturbed throughout anti-TB treatment). This may explain why several genes from our treatment response signature (*e.g.*, *FCGR1A*, *CCL4*, *GBP5*, and *CD3E*) appeared in our treatment outcome signature. However, the magnitude of the treatment response was different: although levels of *FCGR1A* and *GBP5* were decreased throughout anti-TB treatment in patients with a poor treatment outcome, the decrease was delayed (two months versus two weeks) and levels remained higher after six months of treatment compared with patients with a good treatment outcome. *CCL4* expression levels were upregulated in both patient groups upon treatment, but the response was delayed to two months versus two weeks in patients with a poor versus good treatment outcome. Furthermore, the magnitude of the *global* gene expression was different between patients with a poor versus good treatment outcome: whereas patients with a good treatment outcome normalized towards healthy controls regarding molecular degree of perturbation (MDP) scores, patients with a poor treatment outcome did not. Patients with a poor treatment outcome displayed a different transcriptome before the onset of treatment and may therefore require longer or additional treatment to reach a similar clinical endpoint as patients with a good treatment outcome.

Many genes that appeared in our treatment outcome signatures were already described in a published treatment outcome signature (*i.e.*, *CD3E*, *PTPRCv1*, *NOD2*) (20), and in published TB risk signatures (*i.e.*, *GBP1*, *GBP2*, *GBP5*, *FCGR1A*, *STAT1*, *TAP1*) (22, 31). Interestingly, four genes (*i.e.*, *BCL2*, *CCL4*, *PTPRCv1*, and *CD3E*) that appeared in our treatment outcome signatures at diagnosis and week two were recently described to be differentially expressed between deceased and surviving HIV-TB-coinfected patients, and *PTPRCv1* and *CD3E* were among the seven genes that could accurately predict mortality in HIV-TB patients (AUC = 0.86 for each single gene) (32). The occurrence of genes in multiple published gene signatures underscores the validity of our approach in identifying novel biomarkers predicting treatment outcome and suggests that these biomarkers can be applied to TB patients with concomitant DM.

From gene signatures to clinical application: future prospects

To date, numerous transcriptomic signatures for active TB have been reported, but none of them has been developed into a diagnostic point-of care test. Transcriptomic-based diagnostic signatures requires highly specialized and skillful personnel and analytical techniques and is yet not applicable in resource-limited settings. Published gene signatures often contain a large number of transcripts, which is useful for biomarkers discovery, but a TB biomarker signature preferably contains only a few genes to allow application in low-resource settings. Thus, further research is required to develop transcriptomic-based diagnostic assays that can be used at point-of-care facilities.

Several studies have demonstrated the potential of minimal TB signatures. After biomarker identification, reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) can be applied to perform absolute quantification of gene expression, leading to minimal gene signatures. Jacobsen and colleagues performed RT-qPCR following microarray analysis to obtain a three-gene signature (*i.e.*, *Rab33A*, *CD64*, and *LTF*) that discriminated well between TB patients and non-*Mtb*-infected healthy controls or *Mtb*-infected healthy controls with a prediction accuracy of 85% and 80% in an independent validation dataset, respectively (33). Maertzdorf and colleagues used a similar approach to identify minimal gene signatures: they selected genes that were differentially expressed between TB patients and healthy controls in published microarray datasets and they measured the expression of those genes by RT-PCR (34). The identified four-gene signature (*i.e.*, *GBP1*, *ID3*, *P2RY14* and *IFITM3*) showed excellent classification performance of TB by internal cross-validation (AUC = 0.98) and high performance in external datasets (AUC = 0.82-0.89). In addition to the reduction of gene signatures by using RT-qPCR, computational methods can be used to obtain minimal biomarker sets. Roe and colleagues applied machine learning with feature selection on microarray data to

diagnose active pulmonary and extrapulmonary TB based on the transcript levels of a single biomarker (*i.e.*, *BATF2*), with high performance in multiple published cohorts (AUC = 0.93-0.99), and slightly reduced performance in HIV-infected individuals (AUC = 0.85) (35). Gliddon and colleagues also applied feature selection to reduce the number of transcripts of the Kaforou signature (36). In that study, a four-gene signature (*i.e.*, *GBP6*, *TMCC1*, *PRDM1*, and *ARG1*) distinguished TB from other diseases (*i.e.*, pneumonia, lung cancer, Stills disease, Systemic Lupus Erythematosus, *Staphylococcus*, and *Streptococcus*) and a three-gene signature (*i.e.*, *FCGR1A*, *ZNF296*, and *C1QB*) distinguished TB from LTBI with a high performance within their cohort (AUCs = 0.94 and 0.95, respectively) in cross-validation datasets (AUCs = 0.88-0.98 and > 0.91, respectively). Further, data from different cohorts can be combined to generalize gene signatures among heterogeneous populations varying in age, ethnicity, or comorbidities. Sweeney and colleagues performed a multicohort analysis to identify a three-gene signature (*i.e.*, *GBP5*, *DUSP3*, and *KLF2*) that showed robust performance across multiple validation datasets (AUC active TB versus healthy = 0.90; AUC active TB versus LTBI = 0.88) (17). This three-gene signature could distinguish TB progressors from non-progressors (LTBI) at six months prior to positive sputum test (AUC = 0.86) (31). Thus, further studies can employ minimal gene signatures to allow application in TB-endemic areas and facilitate diagnostic test development.

In our cohort studies, we did not study the classification capacity of gene signatures to distinguish TB or TB/DM from other diseases. Transcriptional signatures may represent general host responses, which can be shared among other infectious diseases, thereby reducing the disease specificity of host biomarkers. For example, the blood transcriptome of TB patients was similar to the transcriptome of active sarcoidosis (a granulomatous lung disease with unknown etiology but with many features shared with TB) patients and both transcriptomes were dominated by overexpression of IFN-inducible genes (11, 37). Indeed, many genes that frequently appear in gene signatures for active TB (*e.g.*, *FCGR1A*, *IFI6*, *IFIT3*, *IFITM3*, *GBP1*, *GBP5*, *INDO*, *STAT1*), including our signatures, are also overexpressed in patients with active sarcoidosis (11). TB, however, could be distinguished from sarcoidosis by classification based on discriminatory genes. Thus, gene signatures need to be finetuned to distinguish TB from sarcoidosis (11, 37). Together, these studies suggest that gene signatures could distinguish TB from other diseases, when models are trained on datasets that include data from patients with other diseases.

Despite the large number of identified transcriptomic TB biomarkers in the last decades, gene signatures have not been translated to clinical practice yet, and the diagnostic test performance of published signatures across different populations has only recently been evaluated in independent populations. The WHO proposed minimal and optimal target product profiles (TPP) for a biomarker-based non-sputum-based test to

diagnose TB (38). Minimal TPP includes a sensitivity of at least 65% and a 98% specificity for a confirmatory test (in combination with smear microscopy) and a 95% sensitivity and 80% specificity (depending on TB prevalence) for a point-of-care triage test to identify people who need further testing. Warsinke and colleagues evaluated the potential of 16 reported transcriptomic signatures to diagnose active TB in a pooled dataset, and found that two signatures achieved the minimal WHO TPP for a triage test (39). Furthermore, seven gene signatures could predict progression from LTBI to active TB six months prior to sputum conversion. Gupta and colleagues evaluated the performance of 17 reported transcriptomic signature to diagnose incipient (*i.e.*, the phase between LTBI and active disease) TB in a pooled dataset with samples from four countries (South Africa, Ethiopia, The Gambia, and the UK) (40). Eight signatures met or approximated the minimum TPP for predicting the short-term (0-3 months) risk of TB. In a follow-up meta-analysis, Turner and colleagues extended the inclusion criteria to validate the performance of 27 reported transcriptomic signatures to identify active TB in South African adults (41). Here, four signatures met or approximated the minimal requirements for a triage test, but not for an optimal triage test or a minimal confirmatory test. Finally, Mulenga and colleagues evaluated the performance of 24 signatures for TB diagnosis, and found that four signatures had the potential as diagnostic test (42). Among the aforementioned studies, the Sweeney 3-gene (17), Zak 16-gene (22), Kaforou 25-gene (16), Roe 3-gene (43) and BATF2 (35) gene signatures met the WHO TPP criteria in multiple studies, demonstrating the robustness of these signatures.

Taken together, published host transcriptomic-based biomarker signatures achieve promising sensitivity and specificity rates for a clinical triage test, but current biomarker signatures require further optimization to reach minimal WHO TPP criteria for application as diagnostic TB test. Also, further steps are required to develop low-cost point-of-care assays, that can be used in resource-limited settings. In conclusion, current meta-analysis studies support the research pipeline of transcriptomic-based biomarker discovery using genome-wide approaches for the development of TB diagnostic assays, and suggest that meta-analyses are useful to assess the potential of biomarker signatures as standalone diagnostic tests.

Drug discovery: identification of drug candidates

The second objective of this thesis was to identify novel drug candidates with translational potential for HDT against TB or related intracellular infections such as *Salmonella* bacteria. Drug discovery can follow two pipelines: the drug-to-target or the target-to-drug approach (44). The drug-to-target approach requires screening assays to identify drugs with antimicrobial activity, followed by drug target identification. This approach has been

successfully applied by our laboratory to discover drug candidates with HDT activity against *Mtb* and *Stm* in human cell lines (45, 46). However, considering the polypharmacology of many compounds, identification of the target responsible for the antimicrobial effect is challenging. Alternatively, the target-to-drug approach can be applied, using promising host or pathogenic targets as starting point. The ‘target’ in the target-to-drug approach was originally designed based on a protein, enzyme, or other pathway essential for *Mycobacterium tuberculosis* (*Mtb*) growth or survival *in vitro* or *in vivo*, which was identified by genetic studies using mutant *Mtb* strains, leading to rationally designed inhibitors of the identified *Mtb* essential molecules. In the context of HDT, often a drug-repurposing strategy is followed in which a drug-to-target approach prevails: in this case ‘host effectors’ – which can be host proteins, enzymes, or pathways, are the target. Once a potential drug presumably acting on a target has been functionally identified and the target validated, additional drugs directed at the same target can be identified relatively rapidly.

Genetic inhibition screens can identify promising host targets against *Mtb*. Jayaswal and colleagues screened a commercial siRNA library for *in vitro* activity against 744 kinases and 288 phosphatases in a *Mtb* infection model in the murine J774.1 macrophage line (47). In our laboratory, Korbee and colleagues screened a commercial siKinome library for *in vitro* activity against *Mtb* in a human melanoma cell line (MelJuSo cells), also identifying potential host targets (45). Both studies validated that targets identified by siRNA screens, including the transforming growth factor beta (TGF- β) type-1 receptor (47), and tyrosine-protein host kinases ABL1, BLK, and NTRK1 (45), were druggable by chemical compounds, leading to improved *Mtb* control. Despite the power of such technological approaches, there nevertheless may be discrepancies between transcriptional and protein expression or targeting data. For example, pyruvate dehydrogenase kinase (PDK) 2 was identified as host target during *Mtb* by knockdown of *PDK2* at the genetic level resulting in reduced *Mtb* outgrowth (45), while chemical inhibition of this host target by DCA failed to reduce intracellular *Mtb* in human macrophages (**Chapter 5**). Although there are numerous possible explanations for this discrepancy, including DCA’s affinity for PDK2 versus other PDK isoforms (*i.e.*, PDK1, PDK3 and PDK4) or intrinsic cell-specific effects (*e.g.*, in basal PDK expression/activity or downstream cellular functions) in cell lines versus primary macrophages, the precise reasons for this currently remains elusive. However, in our and others’ experience (48), primary macrophage cultures are challenging to transfect and do not yield high numbers of cells for larger genetic screens, thereby confining the use of larger genetic screens to model human cell lines.

Due to the limitation of genetic screens as mentioned above, we studied the effect of candidate drugs with reported functional abilities to interfere with DM-associated

pathways which, given the reported TB-DM interactions in macrophages (49-53), are likely to interfere with host immunity against *Mtb* at the cellular level. This approach could provide an opportunity to target both TB and DM simultaneously. Since DM is likely to increase glucose uptake and metabolism in macrophages (54), we particularly studied the effect of modulating key metabolic pathways on intracellular *Mtb*. Using this approach, we identified novel host targets involved in the interaction between macrophages and *Mtb*. Also, we compared the response of macrophages to *Salmonella enterica* serovar Typhimurium (*Stm*) versus *Mtb* infection, improving our understanding of the biology of host-pathogen interactions. In the next sections, pathways described in this thesis in the context of previous studies will be summarized and reflected upon, to conclude with promising host targets for HDT.

Murine versus human macrophages in *in vitro* (infection) models

Most studies on macrophage activation and metabolism have been performed using mouse bone-marrow-derived macrophages (BMDMs), but it remains incompletely understood to what extent these findings translate to human macrophages. Several studies have shown that murine and human macrophages respond differently to immunological stimuli and infections with pathogens in the context of metabolism. Stimulation with interleukin (IL)-4 caused only moderate changes of oxidative phosphorylation (OxPhos) and fatty acid oxidation (FAO) in human alternatively activated macrophages (M2), in contrast to mouse BMDMs (55). Importantly, human M2s did not display increased oxidative metabolism compared to human classically activated macrophages (M1s). IL-4 stimulation slightly increased oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) in human M2 without changing the OCR/ECAR ratio (55). Importantly, these data suggest that macrophage activation increases the metabolic flux in human M2 but is not accompanied by a switch from glycolytic to oxidative metabolism. In another study, human M2 did not alter OxPhos in response to lipopolysaccharide (LPS) and slightly reduced glycolysis (56). In contrast, mouse BMDMs polarized to M2 shifted their metabolism from OxPhos to glycolysis in response to LPS. Further, LPS-stimulation induced high lactate levels in mouse BMDMs, but not in human monocyte-derived macrophages (MDMs). In this thesis, human M1 had a higher metabolic flux compared to human M2, but both macrophage subsets increased the ECAR/OCR ratio in response to activation with *Stm* or *Mtb* lysate, again showing an absence of a shift from glycolysis to OxPhos in M2 (**Chapter 5**). Together, these data highlight fundamental differences in the metabolism of polarized macrophages of murine versus human origin, which is summarized in Figure 2. These differences argue for performing studies on the potential of metabolic compounds for HDT in human macrophages.

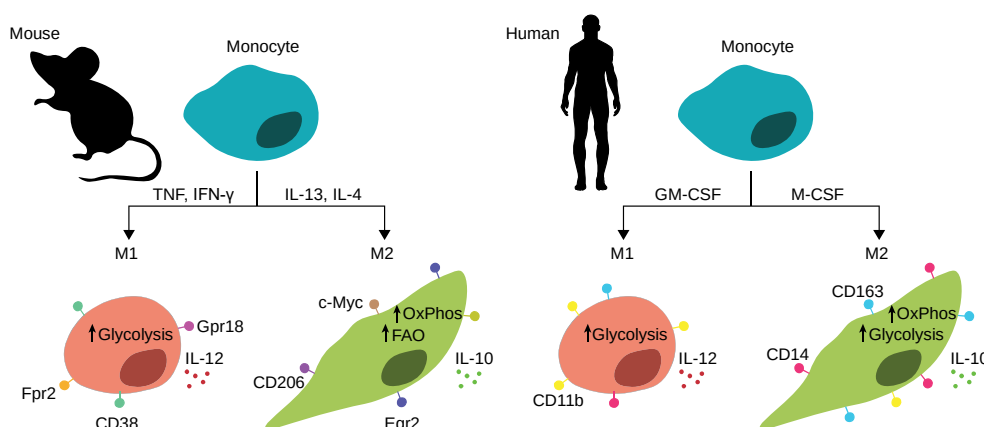


FIGURE 2 | *In vitro* macrophage polarization in human models versus mouse models. Mouse M1 or M2 can be derived from monocytes by TNF- or IFN- γ -induced differentiation or by IL-13- or IL-4-induced differentiation, respectively. Murine M1 and M2 phenotypes are characterized by IL-12 and IL-10 production, and by expression of surface markers (M1: CD38, Fpr2, Gpr19; M2: CD206, c-Myc, Egr2), respectively (48, 57-59). Upon stimulation with bacterial stimuli, *e.g.*, LPS, murine M1 macrophages induce glycolysis, whereas murine M2 macrophages induce oxidative phosphorylation (OxPhos) and fatty acid oxidation (FAO) (48, 60-65). In contrast, human M1 or M2 cells can be derived from monocytes by GM-CSF- or M-CSF-induced differentiation, respectively. Human M1 and M2 phenotypes are characterized by surface markers expression (M1: CD11b^{high}, CD14^{low}, CD163^{low}; M2: CD11b^{low}, CD14^{high}, CD163^{high}) (46, 66). Human M1 and M2 produce IL-12 and IL-10 upon stimulation with bacterial stimuli, respectively, and both macrophage subsets induce glycolysis (Chapter 5).

Central host metabolism

Consistent with induced glycolysis upon M1 activation, transcripts and proteins associated with the Warburg effect were upregulated in *Mtb*-infected mouse and rabbit lungs and in lung granulomas of patients with active TB (67, 68). Glycolytic adenosine triphosphate (ATP) synthesis is less efficient than OxPhos, but it can be induced more rapidly. The Warburg effect's extent may depend on the location inside the granuloma, as reflected by spatial differential expression of Warburg-effect markers in macrophages, with a lower Warburg effect in the center of granulomas compared to the periphery (68).

In this thesis, human macrophages shifted their metabolism towards glycolysis upon stimulation with *Mtb* lysate, in agreement with published data using *Mtb* lysates (69, 70), but not upon infection with live *Mtb* bacteria, also consistent with published data (71, 72) (Chapter 5). This suggests that live but not killed *Mtb* actively suppresses the Warburg effect in host cells. Importantly, human M2s were able to shift their metabolism to glycolysis in the presence of live bacteria. It is speculated that the increase in glycolysis functions rapidly provides energy and biosynthesis of inflammatory molecules during the initial infection stage (73). Gleeson and colleagues indeed showed that the immune

response of human MDMs to *Mtb* was dependent on glycolysis, and inhibition of glycolysis by substitution of glucose for galactose and 2-DG impaired mycobacterial killing by human MDMs and AMs (74). In addition, inhibition of glycolysis by 2-DG inhibited the production of pro-inflammatory cytokines (*i.e.*, TNF- α and IL-12p40) by mouse BMDMs (75). This may explain why human M1s failed to produce IL-12 in response to live *Mtb* infection (**Chapter 4** and **Chapter 5**). Generally, M1s had an attenuated cytokine response to live *Mtb* infection compared to M2s (Figure 3). In contrast, cytokine production was not impaired in M1s upon *Stm* infection (**Chapter 5**). This suggests that the low cytokine production in *Mtb*-infected M1s is not caused by experimental issues, and is in agreement with the observation that glycolysis was not impaired in *Stm*-infected M1. The hypothesis that glycolysis is required for immunity against *Mtb* is further supported by our observation that inhibition of glycolysis by glucose substitution for galactose tended to provide a more beneficial niche for *Mtb* (**Chapter 5**), which has also been detected in human MDMs (74) and mouse BMDMs (76). Remarkably, in contrast to the situation for *Mtb*, glucose substitution for galactose significantly reduced *Stm* levels in human M1 (**Chapter 5**), possibly because *Stm* can utilize glucose as a carbon source (77). This suggests that even when using a similar host niche for persistence and proliferation, *Stm* and *Mtb* may prefer different host metabolic pathways to balance immunometabolism and supply of host nutrients.

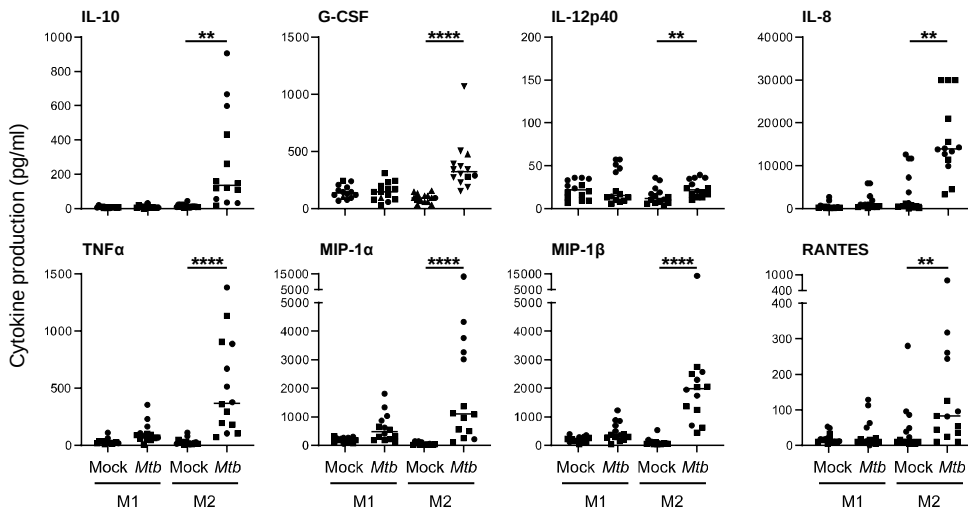


FIGURE 3 | Cytokine and chemokine response of human M1 and M2 upon *Mtb* infection. Cytokines and chemokines measured in the supernatants of *Mtb*-infected macrophages. Differences were significant by non-parametric Friedman's test followed by Dunn's multiple test correction. Adapted from **Chapter 4** and **Chapter 5**.

Together, these studies provide increasing evidence that *Mtb* perturbs the Warburg as a survival strategy (68), potentially interfering with macrophage activation and function (Figure 4). This suggests that compounds that enhance the Warburg effect in host immune cells might be used as HDT against *Mtb* infections (68).

Lactate metabolism

Stimulation of the Warburg effect results in the conversion of pyruvate, the end product of glycolysis, into lactate instead of acetyl-CoA which feeds the tricarboxylic acid (TCA) cycle. This conversion is facilitated by lactate dehydrogenase (LDH), which is expressed upon hypoxia-inducible factor 1-alpha (HIF-1 α) activation (78). The resulting lactate accumulation is a hallmark of the Warburg effect. Additionally, lactate levels are elevated in patients with type 2 DM and potentially reflect chronic inflammation (79, 80). Lactate enhanced Nuclear factor kappa B (NF- κ B) activity and LPS-induced cytokine expression in U937 macrophage-like cells and expression of inflammatory genes in human monocyte-derived macrophages (81, 82).

In addition, lactate has been associated with TB. Lactate accumulation has been detected in *Mtb*-infected guinea pig lung tissue (83) and LDH was upregulated during *Mtb* infection in mouse lungs (67, 84). Upregulation of the Warburg effect in granulomas of TB patients suggests that increased lactate levels may also be present in TB patients (68).

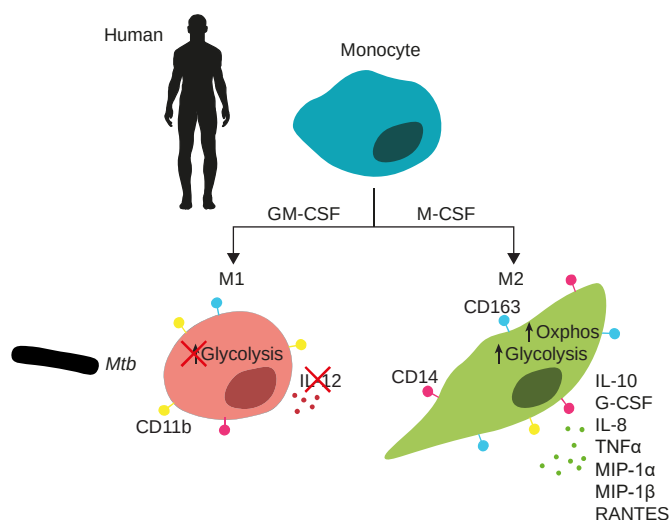


FIGURE 4 | Macrophage response to *Mtb* infection. M1s failed to induce glycolysis (71) (and Chapter 5) and to produce IL-12 in response to *Mtb* (66) (and Chapter 4 and 5), whereas M2s induced glycolysis (Chapter 5) and a cytokine/chemokine response upon *Mtb*-infection (Chapter 4 and 5).

Interestingly, lactate can be consumed by various intracellular bacteria during infection, including *Listeria monocytogenes* (85), *Salmonella enterica* serovar Typhi (86), and *Mtb* (87). High concentrations (10 mM) of lactate promoted *Mtb* growth by functioning as a carbon substrate, while very high concentrations (25-50 mM) of lactate were toxic for *Mtb*, suggesting that the effect of lactate on *Mtb* is concentration-dependent (87). Strikingly, *in vitro* proliferation of *Mtb* mutants lacking IldD2 (encoding lactate dehydrogenase) was impaired compared to wildtype *Mtb* in human monocyte-derived macrophages. These data suggest that *Mtb* may benefit from increased glycolytic pathways by extracting carbon substrates for biomass formation, especially in the early phase of infection and in *in vitro* infection models.

In **Chapter 5**, we detected increased lactate levels in *Mtb*-infected M2, but surprisingly not in *Mtb*-infected M1, again in agreement with inhibition of glycolysis, as discussed above. In *Mtb*-infected M1 and M2, lactate levels did not exceed 10 mM, suggesting that lactate may support *Mtb* growth (87). In support of this hypothesis, the LDH-inhibitor FX-11 reduced the percentage of *Mtb*-infected macrophages (**Chapter 4**). These data suggest that inhibition of lactate production may be used as HDT against *Mtb* infections. Further studies are required to establish lactate levels in TB granulomas and the potential of LDH inhibitors for HDT in the late phase of *Mtb* infections.

Pyruvate metabolism

The glycolytic end product pyruvate can be converted into Acetyl-CoA by pyruvate dehydrogenase (PDH). Pyruvate dehydrogenase kinase (PDK) is an inhibitor of PDH and is a direct target of HIF-1 α to inhibit TCA cycle supply and promote pyruvate conversion into lactate instead (88, 89). PDK levels are elevated in DM patients, and PDK inhibition has been proposed as a therapeutic target for DM (90, 91).

Chemical inhibition of PDKs by DCA treatment resulted in major *Stm*-reducing effects in M1 and M2 but had no decreasing effect on intracellular *Mtb* (**Chapter 5**). Interestingly, mitochondrial AKT has been shown to be an upstream target of PDK1 and is able to phosphorylate PDK1 on threonine (Thr) 346 to inactivate PDH in a kinase assay in hypoxic conditions (92). H-89, a chemical compound that regulates *Stm* outgrowth in a host-directed manner, is a described AKT1 inhibitor (93). Here, we confirm that, like DCA, H-89 is a suitable inhibitor of *Stm*, but not *Mtb*, implying that H-89-induced *Stm* control may be regulated via the AKT1/PDK pathway. It remains to be elucidated whether H-89 treatment results in activation of PDH, like DCA, in our macrophage model.

In our studies, we showed that DCA activated PDH in human M1 and M2, but downstream OxPhos was only detected in human M1, suggesting that the effect of DCA on *Stm* was *not* caused by a shift from glycolysis to OxPhos. We propose that the effect of

DCA on *Stm* is caused by induction of pathways upstream of OxPhos, for example by effects of Acetyl-CoA or TCA cycle intermediates (Figure 5). DCA treatment increased acetyl-CoA levels and TCA cycle intermediates (*i.e.*, citrate, succinate, fumarate, malate, and oxaloacetate) in lung cancer cells (94). Acetyl-CoA can be recycled to pyruvate through ATP-citrate lyase (ACLY)-catalyzed citrate metabolism, which is accompanied by the production of NO, ROS, and NADPH (73). ACLY converts citrate into oxaloacetate (OAA) and acetyl-CoA in the cytosol, which promotes lipid biosynthesis (73). This may explain why we found significantly increased levels of *Mtb* CFUs in human M2 upon DCA treatment (**Chapter 5**) since fatty acids are implicated as the major energy and nutrient source for *Mtb* (77, 95, 96). Alternatively, acetyl-CoA can be converted into ketone body 3-hydroxybutyric acid (3HB) to drive foamy macrophage differentiation (97), which has been linked with *Mtb* virulence in human THP-1 cells by providing a secure host niche and nutrients (97, 98). This suggests that pyruvate metabolism supports *Mtb* growth in contrast to *Stm*. This is consistent with the published finding that suppression of mitochondrial Acetyl-CoA by the mitochondrial pyruvate carrier (MPC) inhibitor UK5099 restricted *Mtb* growth in streptozotocin-induced diabetic mice (98).

Further, citrate may be converted into UDP-acetylglucosamine (UDP-GlcNAc), a component of hyaluronic acid (HA), which has been found to inhibit *Stm* infection in human epithelial cells and reduce colonization of *Stm* in the intestine of mice (99). Interestingly, DCA induced O-GlcNAcylation as well as HA synthesis in a human oesophageal cancer cells line (100), raising the question of whether the protective effects of DCA are regulated via HA, which remains further investigation.

In conclusion, targeting pathways that reduce mitochondrial Acetyl-CoA levels may have the potential as a novel HDT strategy against *Mtb* infections. In contrast, our data suggest that DCA-induced activation of PDH is a promising strategy for HDT against *Stm* infections. The downstream mechanism of action of DCA that is responsible for *Stm* control remains to be determined (Figure 5). In previous studies, others have suggested the presence of additional targets of DCA. DCA was demonstrated to inhibit the PPP in MDA-MB-cancer cells (101) and it has recently been hypothesized that DCA is an antimetabolite that antagonizes the biochemical effects of acetate (102). Thus, the contribution of alternative DCA-induced (metabolic) pathways to protection against *Stm* should be considered.

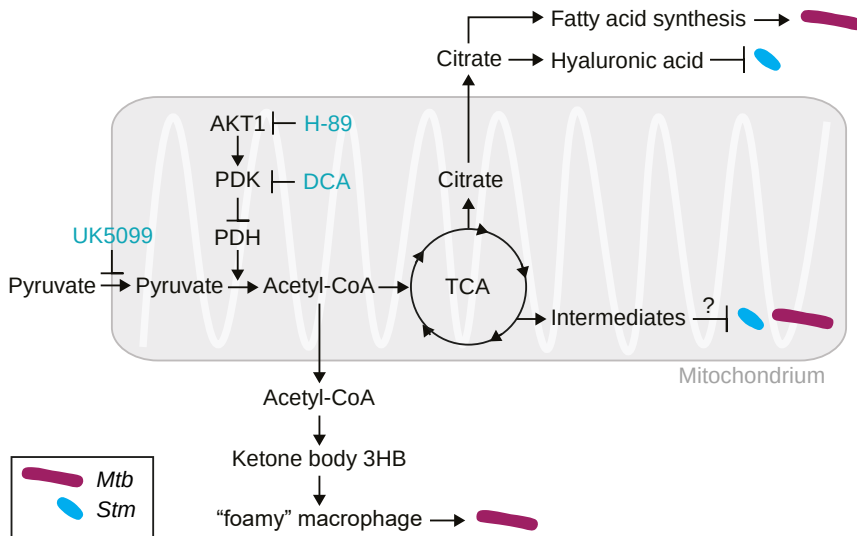


FIGURE 5 | The link between pyruvate metabolism and *Stm* and *Mtb* survival and/or proliferation in human macrophages. Pyruvate metabolism supports intracellular *Mtb* and inhibition of mitochondrial pyruvate and Acetyl-CoA may provide an opportunity for HDT against *Mtb* infections. In contrast, induction of pyruvate metabolism inhibits intracellular *Stm* and may provide an opportunity for HDT against *Stm* infections. Acetyl-CoA can be converted into ketone body 3-hydroxybutyric acid (3HB) to stimulate "foamy" macrophage differentiation (97, 98). TCA cycle intermediate citrate may be exported out of the mitochondria to induce fatty acid synthesis (73), or can be converted into hyaluronic acid (100).

Poly (ADP-ribose) Polymerase (PARP)

Poly (ADP-ribose) Polymerase (PARP) enzymes are involved in DNA repair and programmed cell death. PARP activation has been associated with DM, and mice lacking PARP were resistant to streptozotocin-induced diabetes (103). Excessive oxidative stress leads to PARP overactivation, leading to consequent depletion of its substrate nicotinamide adenine dinucleotide (NAD⁺) and impaired metabolism (glycolysis and oxidative phosphorylation), eventually resulting in ATP depletion (104). In **Chapter 4**, we found that PARP inhibitors induce *Mtb* control in primary human macrophages. NAD⁺ is required for many cellular processes and is required to maintain a pro-inflammatory immune response in macrophages (105). Our data support this hypothesis, as we observed a modified macrophage response to *Mtb* upon treatment with PARP inhibitors, as indicated by altered cytokine and chemokine secretion. Although not studied, maintained NAD⁺ levels could be the mechanism of action of PARP-induced host control against intracellular *Mtb*, which warrants further studies.

Other metabolites

Notably, particular host metabolites are associated with macrophage activation and their immunological responses to LPS and bacterial intracellular pathogens. TCA cycle intermediate citrate can be converted in itaconate, and murine M1-BMDM produces high itaconate levels in response to LPS (in contrast to M2-BMDM) (64, 106). Itaconate-induced anti-inflammatory effects by inhibiting succinate dehydrogenase activity, leading to succinate accumulation. Exogenous itaconate showed direct microbicidal effectivity against *Stm* and *Mtb* (107). Of note, intracellular itaconate levels were much lower in human MDMs compared to RAW264.7 cells (8 mM versus 60 μ M), and it is questionable whether such low concentrations show activity against *Stm* and *Mtb* (107). In addition to itaconate, LPS-induced activation of BMDMs leads to accumulation of succinate, which is a pro-inflammatory TCA cycle intermediate that induces HIF-1 α expression and IL-1 β production (65, 108). Thus, TCA cycle metabolites may regulate macrophage activation and key pro-inflammatory pathways. Hence, other, less well characterized metabolites deserve consideration in the development of HDT against intracellular bacterial infections.

Targeting host metabolic pathways for HDT

In this thesis, we have demonstrated the potential of host metabolic pathways as targets for HDT against intracellular bacterial infections. A schematic overview of macrophage metabolism representing the associations of metabolites with stimulation or inhibition of intracellular *Stm* and *Mtb* survival is shown in Figure 6. Various components of macrophage metabolism have the potential for HDT against *Stm* and *Mtb*, which is summarized in Table 1.

TABLE 1 | Proposed HDT strategies to target host pathways for the treatment of intracellular *Stm* and *Mtb* infections.

HOST TARGET	HDT STRATEGY AGAINST <i>STM</i>	HDT STRATEGY AGAINST <i>MTB</i>
GLYCOLYSIS	Inhibit glycolysis	Enhance the Warburg effect
PYRUVATE METABOLISM	Increase mitochondrial pyruvate and acetyl-CoA levels	Inhibit mitochondrial pyruvate and acetyl-CoA levels
LACTATE PRODUCTION	-	Inhibit pyruvate conversion into lactate; inhibit lactate dehydrogenase
TCA CYCLE	Induce itaconate	Induce itaconate
PARP	-	Inhibit PARP activation

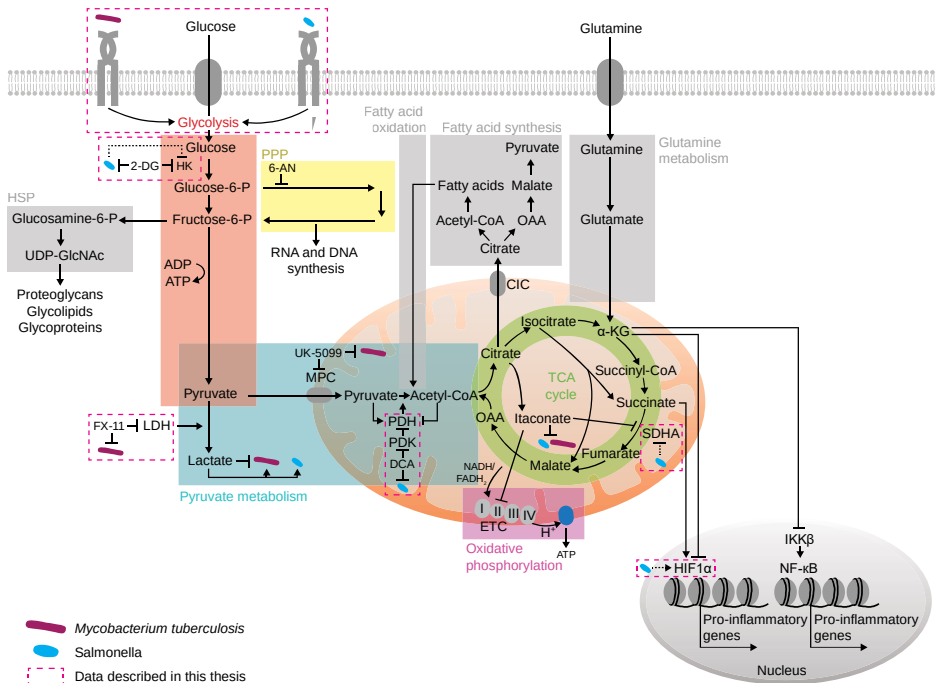


FIGURE 6 | The potential of targeting macrophage metabolic pathways in the context of HDT against *Salmonella* and *Mtb*. Colors indicate main metabolic pathways that are reflected upon in this thesis (glycolysis, PPP, pyruvate metabolism, TCA cycle and oxidative phosphorylation). Associations of (parts of) metabolic pathways with intracellular survival or control of *Stm* or *Mtb* that are supported by data described in this thesis are annotated with dashed squares. Solid arrows indicate protein associations and dashed arrows indicate gene associations. Protein associations are adapted from following publications: (64, 73). Metabolite associations with *Salmonella* and *Mtb* are adapted from following publications: lactate and *Salmonella* (86) or *Mtb* (87); itaconate and *Mtb* (107); UK5099 and *Mtb* (98).

Identification of novel drug candidates for host-directed therapy: future prospects

In this thesis, we have identified host targets and drug candidates that showed activity against intracellular bacteria. Human cell-based screens represent our main approach for drug discovery, and provide knowledge on host-pathogen interactions at least under *in vitro* conditions. However, a significant number of steps need to be taken to translate *in vitro*-identified drug candidates to clinical applications.

First, next to high potency, new anti-TB drugs should have a good safety profile. We have tested the effect of HDT compounds on cellular toxicity, but we did not investigate toxicity effects *in vivo*. Most compounds described in this thesis are repurposed drugs, which are molecules that were clinically used for the treatment of other infections or

diseases. Information on human safety and pharmacokinetics is known for these drugs, which should accelerate their potential translation for other applications, including anti-TB treatment. Whether these drugs are safe in TB patients remains to be investigated.

Second, by definition, *in vitro* conditions differ from *in vivo* conditions, and therefore identified hit compounds may or may not act *in vivo*. For example, novel pyrimidine–imidazoles were identified as potent inhibitors against *Mtb* *in vitro* but lacked activity *in vivo* (109). Discrepancies between *in vitro* and *in vivo* experiments may be caused by culture environments that are not relevant *in vivo* (e.g., culture medium composition) or by *in vivo* pharmacokinetics.

Third, the HDT compounds identified in this thesis showed varying activity against *Stm* and *Mtb* (Table 2). Generally, compounds were more potent against *Stm* compared to *Mtb*, and the efficacy against *Mtb* was relatively low but in the same range as published anti-mycobacterial HDT compounds (Table 3). The limited activity of HDT compounds may be explained by the experimental design. We used a stringent drug discovery model: mycobacteria are exposed to the compounds after overnight infection, which reflects only one generation time. It is expected that a prolonged exposure time may improve the activity of some, but not all, compounds, as exemplified by metformin which did not improve efficacy with 72h stimulation compared to 24h stimulation in human M2 (110). In contrast, several compounds targeting RNA sensor gene expression (*i.e.*, RM5061, NTZ, TIZ) showed no activity with an exposure time of 24 hours, while the antimycobacterial effect was significant after 2, 3, and 4 days of incubation in PBMCs (111). Whether the efficacy of HDT compounds is sufficient for clinical use remains to be investigated.

Finally, since HDT compounds are expected to synergize with antibiotics, we propose the use of HDT compounds as combinatorial therapy with anti-TB antibiotics. Unfortunately, the potential “dual-hit based” synergy of HDT compounds with anti-TB antibiotics is often overlooked in publications. The potential of HDT compounds, however, may be limited by their interaction with antibiotics, as exemplified by rapamycin. Rapamycin is the best studied autophagy inducer, which reduces *Mtb* in mouse BMDMs (112). Unfortunately, rapamycin is metabolized by CYP3A4, a cytochrome P450 enzyme, which is induced by rifampicin treatment (113). Hence, it is essential to consider the interaction of HDT compounds with anti-TB antibiotics.

TABLE 2 | *In vitro* anti-bacterial activity of HDT compounds identified in this thesis

COMPOUND	TARGET	PATHOGEN	CELLULAR MODEL	EXPO- SURE (H)	REDUCTION (%)	CHAPTER
H-89	AKT1	<i>Mtb</i>	M1-M2	24	20-20	4
A-966492	PARP	<i>Mtb</i>	M1-M2	24	10-40	4
NIRAPARIB	PARP	<i>Mtb</i>	M1-M2	24	20-10	4
PAMIPARIB	PARP	<i>Mtb</i>	M1-M2	24	20-10	4
RUCAPARIB	PARP	<i>Mtb</i>	M1-M2	24	30-20	4
FX-11	LDH	<i>Mtb</i>	M1-M2	24	50-20	4
2-DG	G6P	<i>Mtb</i>	M1-M2	24	20-20	5
H-89	AKT1	<i>Stm</i>	M1-M2	24	90-99	4
NIRAPARIB	PARP	<i>Stm</i>	M1-M2	24	60-70	4
DCA	PARP	<i>Stm</i>	M1-M2	24	90-80	5
2-DG	G6P	<i>Stm</i>	M1-M2	24	40-30	5

Abbreviations: 2-DG, 2-deoxy-D-glucose; G6P, glucose-6-phosphate; PARP, poly (ADP-ribose) polymerase

Concluding remarks

In this thesis, we identified host transcriptomic biomarkers to detect and predict response and outcome to anti-TB treatment in TB patients with or without concomitant DM and hyperglycaemia. Furthermore, we identified pharmacological compounds with activity against *Mtb* and *Stm* in a host-directed manner and also identified promising host targets that are druggable by HDT, including host metabolic markers that relate to DM.

The next challenges will be to translate the potential of host-based biomarkers to clinical point-of-care tests and to translate the potential of HDT compounds identified in *in vitro* macrophage assays to *in vivo* studies and, ultimately, to clinical studies.

TABLE 3 | *In vitro* anti-mycobacterial activity of compounds in murine and human macrophages

COMPOUND	TARGET	CELLULAR MODEL	EXPOSURE (H)	CFU REDUCTION (%)	REF
C40.T4	CD40 and TLR4	BMDM	24	28	(114)
IMATINIB	Abl PTK	AM	24	50	(115)
AT9283	RTKs	M1-M2	24	75-45	(45)
ENMD-2076	RTKs	M1-M2	24	75-50	(45)
DOVITINIB	RTKs	M1-M2	24	75-60	(45)
H-89	AKT1	M1-M2	24	25-20	(45)
RM5061	RNA sensor gene expression	PBMC	24	0	(111)
NTZ	RNA sensor gene expression	PBMC	24	0	(111)
TIZ	RNA sensor gene expression	PBMC	24	0	(111)
IBRUTINIB	Autophagy	THP-1	24	50	(116)
METFORMIN	AMPK-activating antidiabetic drug	M2	24	50	(110)
LOPERAMIDE	Autophagy	AM	24	50	(117)
VERAPAMIL	Autophagy	AM	24	88	(117)
EZETIMIBE	2-azetidinone cholesterol absorption	M1	24	40	(118)
EZETIMIBE	2-azetidinone cholesterol absorption	THP-1	24	80	(118)
SIMVASTATIN	Cholesterol accumulation	BMDM	24	33	(119)
H-89	AKT1	M1-M2	24	10-20	(46)
FLUSPIRILENE	Autophagy	M1-M2	24	25-30	(46)
PIMOZIDE	Autophagy	M1-M2	24	30-30	(46)
RAPAMYCIN	mTORC1	M2	24	10	(120)
C4.T4	CLEC4E and TLR4	BMDM	48	83	(121)
N-ACETYL CYSTEINE	ROS scavenger	THP-1	48	30	(122)
TMP195	HDACs	M1-M2	48	5-15	(123)
TMP269	HDACs	M1-M2	48	5-20	(123)
TSA	HDACs	M1-M2	48	25-25	(123)
1,25D3	Vitamin D3 receptor	THP-1	72	20	(124)
4-PHENYL-BUTYRATE	Multiple activities	M1	72	29	(125)
4-PHENYL-BUTYRATE	Multiple activities	M2	72	60	(126)
LL-37	Multiple, including vitamin D-induced autophagy	M2	72	50	(126)
1,25D3	Vitamin D3 receptor	M2	72	60	(126)
RAPAMYCIN	Autophagy	M2	72	75	(126)
ALL-TRANS RETINOIC ACID	Vitamin A metabolite	MDM	72	50	(127)
BAZEDOXIFENE	Selective estrogen receptor	THP-1	72	70	(128)

Abbreviations: Abl, Abelson; CLEC4E, C-Type Lectin Domain Family 4 Member E; HDACs, Histone Deacetylases; mTORC1, mammalian target of rapamycin complex 1; PTK, protein tyrosine kinases; RTK, Receptor tyrosine kinases; TLR4, Toll-like receptor 4.

References

1. Kalra R, Tiwari D, Dkhar HK, Bhagyaraj E, Kumar R, Bhardwaj A, et al. Host factors subverted by *Mycobacterium tuberculosis*: Potential targets for host directed therapy. *International Reviews of Immunology*. 2021;1-28.
2. Walzl G, McNerney R, du Plessis N, Bates M, McHugh TD, Chegou NN, et al. Tuberculosis: advances and challenges in development of new diagnostics and biomarkers. *The Lancet Infectious Diseases*. 2018;18(7):e199-e210.
3. Eckold C, Kumar V, Weiner J, Alisjahbana B, Riza A-L, Ronacher K, et al. Impact of Intermediate Hyperglycemia and Diabetes on Immune Dysfunction in Tuberculosis. *Clinical Infectious Diseases*. 2021;72(1):69-78.
4. Huangfu P, Ugarte-Gil C, Golub J, Pearson F, Critchley J. The effects of diabetes on tuberculosis treatment outcomes: an updated systematic review and meta-analysis. *Int J Tuberc Lung Dis*. 2019;23(7):783-96.
5. Prada-Medina CA, Fukutani KF, Pavan Kumar N, Gil-Santana L, Babu S, Lichtenstein F, et al. Systems immunology of diabetes-tuberculosis comorbidity reveals signatures of disease complications. *Scientific reports*. 2017;7(1):1-16.
6. Grayson BL, Wang L, Aune TM. Peripheral blood gene expression profiles in metabolic syndrome, coronary artery disease and type 2 diabetes. *Genes & Immunity*. 2011;12(5):341-51.
7. Sloot R, van der Loeff MFS, van Zwet EW, Haks MC, Keizer ST, Scholing M, et al. Biomarkers can identify pulmonary tuberculosis in HIV-infected drug users months prior to clinical diagnosis. *EBioMedicine*. 2015;2(2):172-9.
8. Berry MP, Graham CM, McNab FW, Xu Z, Bloch SA, Oni T, et al. An interferon-inducible neutrophil-driven blood transcriptional signature in human tuberculosis. *Nature*. 2010;466(7309):973-7.
9. Maertzdorf J, Ota M, Repsilber D, Mollenkopf HJ, Weiner J, Hill PC, et al. Functional correlations of pathogenesis-driven gene expression signatures in tuberculosis. *PloS one*. 2011;6(10):e26938.
10. Ottenhoff THM, Dass RH, Yang N, Zhang MM, Wong HEE, Sahiratmadja E, et al. Genome-wide expression profiling identifies type 1 interferon response pathways in active tuberculosis. *PLoS one*. 2012;7(9):e45839.
11. Bloom CI, Graham CM, Berry MPR, Rozakeas F, Redford PS, Wang Y, et al. Transcriptional blood signatures distinguish pulmonary tuberculosis, pulmonary sarcoidosis, pneumonias and lung cancers. *PloS one*. 2013;8(8):e70630.
12. Joosten SA, Fletcher HA, Ottenhoff THM. A helicopter perspective on TB biomarkers: pathway and process based analysis of gene expression data provides new insight into TB pathogenesis. *PloS one*. 2013;8(9):e73230.
13. Gebremicael G, Kassa D, Quinten E, Alemayehu Y, Gebreegziavier A, Belay Y, et al. Host gene expression kinetics during treatment of tuberculosis in HIV-coinfected individuals is independent of highly active antiretroviral therapy. *The Journal of infectious diseases*. 2018;218(11):1833-46.
14. Joosten SA, Goeman JJ, Sutherland JS, Opmeer L, De Boer KG, Jacobsen M, et al. Identification of biomarkers for tuberculosis disease using a novel dual-color RT–MLPA assay. *Genes & Immunity*. 2012;13(1):71-82.
15. Bloom CI, Graham CM, Berry MPR, Wilkinson KA, Oni T, Rozakeas F, et al. Detectable changes in the blood transcriptome are present after two weeks of antituberculosis therapy. *PLoS one* 2012;7(10): e46191.

16. Kaforou M, Wright VJ, Oni T, French N, Anderson ST, Bangani N, et al. Detection of tuberculosis in HIV-infected and-uninfected African adults using whole blood RNA expression signatures: a case-control study. *PLoS medicine*. 2013;10(10):e1001538.
17. Sweeney TE, Braviak L, Tato CM, Khatri P. Genome-wide expression for diagnosis of pulmonary tuberculosis: a multicohort analysis. *The Lancet Respiratory Medicine*. 2016;4(3):213-24.
18. Gjøen JE, Jenum S, Sivakumaran D, Mukherjee A, Macaden R, Kabra SK, et al. Novel transcriptional signatures for sputum-independent diagnostics of tuberculosis in children. *Scientific reports*. 2017;7(1):1-9.
19. Thompson EG, Du Y, Malherbe ST, Shankar S, Braun J, Valvo J, et al. Host blood RNA signatures predict the outcome of tuberculosis treatment. *Tuberculosis*. 2017;107:48-58.
20. Sivakumaran D, Jenum S, Vaz M, Selvam S, Ottenhoff THM, Haks MC, et al. Combining host-derived biomarkers with patient characteristics improves signature performance in predicting tuberculosis treatment outcomes. *Communications biology*. 2020;3(1):1-10.
21. Burel JG, Singhania A, Dubelko P, Muller J, Tanner R, Parizotto E, et al. Distinct blood transcriptomic signature of treatment in latent tuberculosis infected individuals at risk of developing active disease. *Tuberculosis*. 2021;131:102127.
22. Zak DE, Penn-Nicholson A, Scriba TJ, Thompson E, Suliman S, Amon LM, et al. A prospective blood RNA signature for tuberculosis disease risk. *J Lancet*. 2016;387(10035):2312.
23. Saunders BM, Britton WJ, biology c. Life and death in the granuloma: immunopathology of tuberculosis. *Immunology and cell biology*. 2007;85(2):103-11.
24. Miotto D, Christodoulouopoulos P, Olivenstein R, Taha R, Cameron L, Tsicopoulos A, et al. Expression of IFN- γ -inducible protein; monocyte chemotactic proteins 1, 3, and 4; and eotaxin in TH1-and TH2-mediated lung diseases. *Journal of Allergy and Clinical Immunology* 2001;107(4):664-70.
25. Kumar NP, Sridhar R, Nair D, Banurekha VV, Nutman TB, Babu S. Type 2 diabetes mellitus is associated with altered CD 8+ T and natural killer cell function in pulmonary tuberculosis. *Immunology*. 2015;144(4):677-86.
26. Kumar NP, Sridhar R, Banurekha VV, Jawahar MS, Fay MP, Nutman TB, et al. Type 2 diabetes mellitus coincident with pulmonary tuberculosis is associated with heightened systemic type 1, type 17, and other proinflammatory cytokines. *Annals of the American Thoracic Society*. 2013;10(5):441-9.
27. De Taeye BM, Novitskaya T, McGuinness OP, Gleaves L, Medda M, Covington JW, et al. Macrophage TNF- α contributes to insulin resistance and hepatic steatosis in diet-induced obesity. *American Journal of Physiology-Endocrinology and Metabolism*. 2007;293(3):E713-E25.
28. Jain A, Singh JA. Harms of TNF inhibitors in rheumatic diseases: a focused review of the literature. *Immunotherapy*. 2013;5(3):265-99.
29. Wei S, Zhang L, Zhou X, Du M, Jiang Z, Hausman GJ, et al. Emerging roles of zinc finger proteins in regulating adipogenesis. *Cellular and molecular life sciences*. 2013;70(23):4569-84.
30. Suchy-Dicey A, Heckbert SR, Smith NL, McKnight B, Rotter JI, Chen YDI, et al. Gene expression in thiazide diuretic or statin users in relation to incident type 2 diabetes. *International Journal of Molecular Epidemiology and Genetics*. 2014;5(1):22.
31. Warsinske HC, Rao AM, Moreira FMF, Santos PCP, Liu AB, Scott M, et al. Assessment of validity of a blood-based 3-gene signature score for progression and diagnosis of tuberculosis, disease severity, and treatment response. *JAMA network open*. 2018;1(6):e183779-e.
32. Gebremicael G, Gebreegziaxier A, Kassa D. Low Transcriptomic of PTPRCv1 and CD3E Is an Independent Predictor of Mortality in HIV and Tuberculosis Co-infected Patient. *Preprint available at Research Square* (<https://doi.org/10.21203/rs.3.rs-1253897/v1>). 2022.

33. Jacobsen M, Repsilber D, Gutschmidt A, Neher A, Feldmann K, Mollenkopf HJ, et al. Candidate biomarkers for discrimination between infection and disease caused by *Mycobacterium tuberculosis*. *Journal of molecular medicine*. 2007;85(6):613-21.
34. Maertzdorf J, McEwen G, Weiner 3rd J, Tian S, Lader E, Schriek U, et al. Concise gene signature for point-of-care classification of tuberculosis. *EMBO molecular medicine*. 2016;8(2):86-95.
35. Roe JK, Thomas N, Gil E, Best K, Tsaliki E, Morris-Jones S, et al. Blood transcriptomic diagnosis of pulmonary and extrapulmonary tuberculosis. *JCI insight*. 2016;1(16).
36. Gliddon HD, Kaforou M, Alikian M, Habgood-Coote D, Zhou C, Oni T, et al. Identification of Reduced Host Transcriptomic Signatures for Tuberculosis Disease and Digital PCR-Based Validation and Quantification. *Frontiers in immunology*. 2021;12:433.
37. Maertzdorf J, Weiner J, Mollenkopf H-J, Network T, Bauer T, Prasse A, et al. Common patterns and disease-related signatures in tuberculosis and sarcoidosis. *Proceedings of the National Academy of Sciences*. 2012;109(20):7853-8.
38. World Health Organization. High priority target product profiles for new tuberculosis diagnostics: report of a consensus meeting, 28-29 April 2014, Geneva, Switzerland. Geneva: World Health Organization; 2014. Contract No.: WHO/HTM/TB/2014.18.
39. Warsinske H, Vashisht R, Khatri P. Host-response-based gene signatures for tuberculosis diagnosis: A systematic comparison of 16 signatures. *PLoS medicine*. 2019;16(4):e1002786.
40. Gupta RK, Turner CT, Venturini C, Esmail H, Rangaka MX, Copas A, et al. Concise whole blood transcriptional signatures for incipient tuberculosis: a systematic review and patient-level pooled meta-analysis. *The Lancet Respiratory medicine*. 2020;8(4):395-406.
41. Turner CT, Gupta RK, Tsaliki E, Roe JK, Mondal P, Nyawo GR, et al. Blood transcriptional biomarkers for active pulmonary tuberculosis in a high-burden setting: a prospective, observational, diagnostic accuracy study. *The Lancet Respiratory Medicine*. 2020;8(4):407-19.
42. Mulenga H, Zaichenberger C-Z, Bunyasi EW, Mbandi SK, Mendelsohn SC, Kagina B, et al. Performance of diagnostic and predictive host blood transcriptomic signatures for Tuberculosis disease: A systematic review and meta-analysis. *PLoS one*. 2020;15(8):e0237574.
43. Roe J, Venturini C, Gupta RK, Gurry C, Chain BM, Sun Y, et al. Blood transcriptomic stratification of short-term risk in contacts of tuberculosis. *Clinical Infectious Diseases*. 2020;70(5):731-7.
44. Sala C, Hartkoorn RC. Tuberculosis drugs: new candidates and how to find more. *Future microbiology*. 2011;6(6):617-33.
45. Korbee CJ, Heemskerk MT, Kocev D, van Strijen E, Rabiee O, Franken KLMC, et al. Combined chemical genetics and data-driven bioinformatics approach identifies receptor tyrosine kinase inhibitors as host-directed antimicrobials. *Nature communications*. 2018;9(1):1-14.
46. Heemskerk MT, Korbee CJ, Esselink JJ, dos Santos CC, van Veen S, Gordijn IF, et al. Repurposing diphenylbutylpiperidine-class antipsychotic drugs for host-directed therapy of *Mycobacterium tuberculosis* and *Salmonella enterica* infections. *Scientific Reports*. 2021;11(1):19634.
47. Jayaswal S, Kamal MA, Dua R, Gupta S, Majumdar T, Das G, et al. Identification of host-dependent survival factors for intracellular *Mycobacterium tuberculosis* through an siRNA screen. *PLoS pathogens*. 2010;6(4):e1000839.
48. Rodríguez-Prados J-C, Través PG, Cuenca J, Rico D, Aragonés J, Martín-Sanz P, et al. Substrate fate in activated macrophages: a comparison between innate, classic, and alternative activation. *The Journal of Immunology*. 2010;185(1):605-14.
49. Lopez-Lopez N, Martinez AGR, Garcia-Hernandez MH, Hernandez-Pando R, Castañeda-Delgado JE, Lugo-Villarino G, et al. Type-2 diabetes alters the basal phenotype of human macrophages and diminishes their capacity to respond, internalise, and control *Mycobacterium tuberculosis*. *Memórias do Instituto Oswaldo Cruz*. 2018;113(4).
50. Restrepo BI, Twahirwa M, Rahbar MH, Schlesinger LS. Phagocytosis via complement or Fc-gamma receptors is compromised in monocytes from type 2 diabetes patients with chronic hyperglycemia. *PLoS one*. 2014;9(3):e92977.

51. Gomez DI, Twahirwa M, Schlesinger LS, Restrepo BI. Reduced *Mycobacterium tuberculosis* association with monocytes from diabetes patients that have poor glucose control. *Tuberculosis*. 2013;93(2):192-7.
52. Valtierra-Alvarado MA, Delgado JEC, Ramírez-Talavera SI, Lugo-Villarino G, Dueñas-Arteaga F, Lugo-Sánchez A, et al. Type 2 diabetes mellitus metabolic control correlates with the phenotype of human monocytes and monocyte-derived macrophages. *Journal of Diabetes and its Complications*. 2020;34(11):107708.
53. Valtierra-Alvarado MA, Lugo-Villarino G, Dueñas-Arteaga F, González-Contreras BE, Lugo-Sánchez A, Castañeda-Delgado JE, et al. Impact of type 2 diabetes on the capacity of human macrophages infected with *Mycobacterium tuberculosis* to modulate monocyte differentiation through a bystander effect. *Immunology and Cell Biology*. 2021;99(10):1026-39.
54. Nishizawa T, Bornfeldt KE. Diabetic vascular disease and the potential role of macrophage glucose metabolism. *Annals of Medicine*. 2012;44(6):555-63.
55. Namgaladze D, Brüne B. Fatty acid oxidation is dispensable for human macrophage IL-4-induced polarization. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids*. 2014;1841(9):1329-35.
56. Vijayan V, Pradhan P, Braud L, Fuchs HR, Gueler F, Motterlini R, et al. Human and murine macrophages exhibit differential metabolic responses to lipopolysaccharide-A divergent role for glycolysis. *Redox biology*. 2019;22:101147.
57. Jablonski KA, Amici SA, Webb LM, Ruiz-Rosado JdD, Popovich PG, Partida-Sanchez S, et al. Novel markers to delineate murine M1 and M2 macrophages. *PloS one*. 2015;10(12):e0145342.
58. Orecchioni M, Ghosheh Y, Pramod AB, Ley K. Macrophage polarization: different gene signatures in M1 (LPS+) vs. classically and M2 (LPS-) vs. alternatively activated macrophages. *Frontiers in immunology*. 2019;10:1084.
59. Fernando MR, Reyes JL, Iannuzzi J, Leung G, McKay DM. The pro-inflammatory cytokine, interleukin-6, enhances the polarization of alternatively activated macrophages. *PloS one*. 2014;9(4):e94188.
60. Vats D, Mukundan L, Odegaard JI, Zhang L, Smith KL, Morel CR, et al. Oxidative metabolism and PGC-1 β attenuate macrophage-mediated inflammation. *Cell metabolism*. 2006;4(1):13-24.
61. Huang SC-C, Everts B, Ivanova Y, O'Sullivan D, Nascimento M, Smith AM, et al. Cell-intrinsic lysosomal lipolysis is essential for alternative activation of macrophages. *Nature immunology*. 2014;15(9):846-55.
62. Freerman AJ, Johnson AR, Sacks GN, Milner JJ, Kirk EL, Troester MA, et al. Metabolic reprogramming of macrophages: glucose transporter 1 (GLUT1)-mediated glucose metabolism drives a proinflammatory phenotype. *Journal of Biological Chemistry*. 2014;289(11):7884-96.
63. Haschemi A, Kosma P, Gille L, Evans CR, Burant CF, Starkl P, et al. The sedoheptulose kinase CARKL directs macrophage polarization through control of glucose metabolism. *Cell metabolism*. 2012;15(6):813-26.
64. Jha AK, Huang SC-C, Sergushichev A, Lampropoulou V, Ivanova Y, Loginicheva E, et al. Network integration of parallel metabolic and transcriptional data reveals metabolic modules that regulate macrophage polarization. *Immunity*. 2015;42(3):419-30.
65. Tannahill GM, Curtis AM, Adamik J, Palsson-McDermott EM, McGettrick AF, Goel G, et al. Succinate is an inflammatory signal that induces IL-1 β through HIF-1 α . *Nature*. 2013;496(7444):238-42.
66. Verreck FAW, de Boer T, Langenberg DML, Hoeve MA, Kramer M, Vaisberg E, et al. Human IL-23-producing type 1 macrophages promote but IL-10-producing type 2 macrophages subvert

- immunity to (myco) bacteria. *Proceedings of the National Academy of Sciences*. 2004;101(13):4560-5.
67. Shi L, Salamon H, Eugenin EA, Pine R, Cooper A, Gennaro ML. Infection with *Mycobacterium tuberculosis* induces the Warburg effect in mouse lungs. *Scientific reports*. 2015;5(1):1-13.
 68. Shi L, Eugenin EA, Subbian S. Immunometabolism in tuberculosis. *Frontiers in immunology*. 2016;7:150.
 69. Lachmandas E, Beigier-Bompadre M, Cheng SC, Kumar V, van Laarhoven A, Wang X, et al. Rewiring cellular metabolism via the AKT/mTOR pathway contributes to host defence against *Mycobacterium tuberculosis* in human and murine cells. *European journal of immunology*. 2016;46(11):2574-86.
 70. Vrieling F, Kostidis S, Spaink HP, Haks MC, Mayboroda OA, Ottenhoff THM, et al. Analyzing the impact of *Mycobacterium tuberculosis* infection on primary human macrophages by combined exploratory and targeted metabolomics. *Scientific reports*. 2020;10(1):1-13.
 71. Cumming BM, Addicott KW, Adamson JH, Steyn AJC. *Mycobacterium tuberculosis* induces decelerated bioenergetic metabolism in human macrophages. *Elife*. 2018;7:e39169.
 72. Hackett EE, Charles-Messance H, O'Leary SM, Gleeson LE, Muñoz-Wolf N, Case S, et al. *Mycobacterium tuberculosis* limits host glycolysis and IL-1 β by restriction of PFK-M via MicroRNA-21. *Cell reports*. 2020;30(1):124-36.
 73. Kelly B, O'Neill LA. Metabolic reprogramming in macrophages and dendritic cells in innate immunity. *Cell research*. 2015;25(7):771-84.
 74. Gleeson LE, Sheedy FJ, Pálsson-McDermott EM, Triglia D, O'Leary SM, O'Sullivan MP, et al. Cutting edge: *Mycobacterium tuberculosis* induces aerobic glycolysis in human alveolar macrophages that is required for control of intracellular bacillary replication. *The Journal of Immunology*. 2016;196(6):2444-9.
 75. Yu Q, Wang Y, Dong L, He Y, Liu R, Yang Q, et al. Regulations of glycolytic activities on macrophages functions in tumor and infectious inflammation. *Frontiers in cellular and infection microbiology*. 2020;10:287.
 76. Braverman J, Sogi KM, Benjamin D, Nomura DK, Stanley SA. HIF-1 α is an essential mediator of IFN- γ -dependent immunity to *Mycobacterium tuberculosis*. *The Journal of Immunology*. 2016;197(4):1287-97.
 77. Eisenreich W, Dandekar T, Heesemann J, Goebel W. Carbon metabolism of intracellular bacterial pathogens and possible links to virulence. *Nature Reviews Microbiology*. 2010;8(6):401-12.
 78. Semenza GL, Jiang B-H, Leung SW, Passantino R, Concordet J-P, Maire P, et al. Hypoxia response elements in the aldolase A, enolase 1, and lactate dehydrogenase A gene promoters contain essential binding sites for hypoxia-inducible factor 1. *Journal of Biological Chemistry*. 1996;271(51):32529-37.
 79. Digirolamo M, Newby FD, Lovejoy J. Lactate production in adipose tissue; a regulated function with extra-adipose implications. *The FASEB Journal*. 1992;6(7):2405-12.
 80. Juraschek SP, Shantha GPS, Chu AY, Miller Iii ER, Guallar E, Hoogeveen RC, et al. Lactate and risk of incident diabetes in a case-cohort of the atherosclerosis risk in communities (ARIC) study. *PloS one*. 2013;8(1):e55113.
 81. Samuvel DJ, Sundararaj KP, Nareika A, Lopes-Virella MF, Huang Y. Lactate boosts TLR4 signaling and NF- κ B pathway-mediated gene transcription in macrophages via monocarboxylate transporters and MD-2 up-regulation. *The Journal of Immunology*. 2009;182(4):2476-84.
 82. Nareika A, He L, Game BA, Slate EH, Sanders JJ, London SD, et al. Sodium lactate increases LPS-stimulated MMP and cytokine expression in U937 histiocytes by enhancing AP-1 and NF- κ B transcriptional activities. *American Journal of Physiology-Endocrinology and Metabolism*. 2005;289(4):E534-E42.

83. Somashekar BS, Amin AG, Rithner CD, Troudt J, Basaraba R, Izzo A, et al. Metabolic profiling of lung granuloma in *Mycobacterium tuberculosis* infected guinea pigs: ex vivo 1H magic angle spinning NMR studies. *Journal of proteome research*. 2011;10(9):4186-95.
84. Domingo-Gonzalez R, Das S, Griffiths KL, Ahmed M, Bambouskova M, Gopal R, et al. Interleukin-17 limits hypoxia-inducible factor 1 α and development of hypoxic granulomas during tuberculosis. *JCI insight*. 2017;2(19).
85. Grubmüller S, Schauer K, Goebel W, Fuchs TM, Eisenreich W. Analysis of carbon substrates used by *Listeria monocytogenes* during growth in J774A. 1 macrophages suggests a bipartite intracellular metabolism. *Frontiers in cellular and infection microbiology*. 2014;4:156.
86. Chai LC, Kong BH, Elemfareji OI, Thong KL. Variable carbon catabolism among *Salmonella enterica* serovar Typhi isolates. *PLoS One*. 2012;7(5):e36201.
87. Billig S, Schneefeld M, Huber C, Grassl GA, Eisenreich W, Bange F-C. Lactate oxidation facilitates growth of *Mycobacterium tuberculosis* in human macrophages. *Scientific reports*. 2017;7(1):1-12.
88. Kim J-w, Tchernyshyov I, Semenza GL, Dang CV. HIF-1-mediated expression of pyruvate dehydrogenase kinase: a metabolic switch required for cellular adaptation to hypoxia. *Cell metabolism*. 2006;3(3):177-85.
89. Papandreou I, Cairns RA, Fontana L, Lim AL, Denko NC. HIF-1 mediates adaptation to hypoxia by actively downregulating mitochondrial oxygen consumption. *Cell metabolism*. 2006;3(3):187-97.
90. Stacpoole PW, Moore GW, Kornhauser DM. Metabolic effects of dichloroacetate in patients with diabetes mellitus and hyperlipoproteinemia. *New England Journal of Medicine*. 1978;298(10):526-30.
91. Jeoung NH. Pyruvate dehydrogenase kinases: therapeutic targets for diabetes and cancers. *Diabetes & metabolism journal*. 2015;39(3):188-97.
92. Chae YC, Vaira V, Caino MC, Tang H-Y, Seo JH, Kossenkova AV, et al. Mitochondrial Akt regulation of hypoxic tumor reprogramming. *Cancer cell*. 2016;30(2):257-72.
93. Kuijl C, Savage NDL, Marsman M, Tuin AW, Janssen L, Egan DA, et al. Intracellular bacterial growth is controlled by a kinase network around PKB/AKT1. *Nature*. 2007;450(7170):725-30.
94. Zhang W, Hu X, Zhou W, Tam KY. Liquid chromatography–tandem mass spectrometry method revealed that lung cancer cells exhibited distinct metabolite profiles upon the treatment with different pyruvate dehydrogenase kinase inhibitors. *Journal of proteome research*. 2018;17(9):3012-21.
95. Schnappinger D, Ehrt S, Voskuil MI, Liu Y, Mangan JA, Monahan IM, et al. Transcriptional adaptation of *Mycobacterium tuberculosis* within macrophages: insights into the phagosomal environment. *The Journal of experimental medicine*. 2003;198(5):693-704.
96. McKinney JD, Zu Bentrup KH, Munoz-Elias EJ, Miczak A, Chen B, Chan W-T, et al. Persistence of *Mycobacterium tuberculosis* in macrophages and mice requires the glyoxylate shunt enzyme isocitrate lyase. *Nature*. 2000;406(6797):735-8.
97. Singh V, Jamwal S, Jain R, Verma P, Gokhale R, Rao KVS. *Mycobacterium tuberculosis*-driven targeted recalibration of macrophage lipid homeostasis promotes the foamy phenotype. *Cell host & microbe*. 2012;12(5):669-81.
98. Mehrotra P, Jamwal SV, Saquib N, Sinha N, Siddiqui Z, Manivel V, et al. Pathogenicity of *Mycobacterium tuberculosis* is expressed by regulating metabolic thresholds of the host macrophage. *PLoS pathogens*. 2014;10(7):e1004265.
99. Kessler SP, Obery DR, Nickerson KP, Petrey AC, McDonald C, de la Motte CA. Multifunctional role of 35 kilodalton hyaluronan in promoting defense of the intestinal epithelium. *Journal of Histochemistry & Cytochemistry*. 2018;66(4):273-87.

100. Twarock S, Reichert C, Bach K, Reiners O, Kretschmer I, Gorski DJ, et al. Inhibition of the hyaluronan matrix enhances metabolic anticancer therapy by dichloroacetate in vitro and in vivo. *British journal of pharmacology*. 2019;176(23):4474-90.
101. De Preter G, Neveu M-A, Danhier P, Brisson L, Payen VL, Porporato PE, et al. Inhibition of the pentose phosphate pathway by dichloroacetate unravels a missing link between aerobic glycolysis and cancer cell proliferation. *Oncotarget*. 2016;7(3):2910.
102. El Sayed SM, Baghdadi H, Ahmed NS, Almaramhy HH, Mahmoud AA-A, El-Sawy SA, et al. Dichloroacetate is an antimetabolite that antagonizes acetate and deprives cancer cells from its benefits: A novel evidence-based medical hypothesis. *Medical hypotheses*. 2019;122:206-9.
103. Burkart V, Wang Z-Q, Radons J, Heller B, Herceg Z, Stingl L, et al. Mice lacking the poly (ADP-ribose) polymerase gene are resistant to pancreatic beta-cell destruction and diabetes development induced by streptozocin. *Nature medicine*. 1999;5(3):314-9.
104. Pacher P, Szabó C. Role of poly (ADP-ribose) polymerase 1 (PARP-1) in cardiovascular diseases: the therapeutic potential of PARP inhibitors. *Cardiovascular drug reviews*. 2007;25(3):235-60.
105. Billingham LK, Chandel NS. NAD-biosynthetic pathways regulate innate immunity. *Nature Immunology*. 2019;20(4):380-2.
106. Lampropoulou V, Sergushichev A, Bambouskova M, Nair S, Vincent EE, Loginicheva E, et al. Itaconate links inhibition of succinate dehydrogenase with macrophage metabolic remodeling and regulation of inflammation. *Cell metabolism*. 2016;24(1):158-66.
107. Michelucci A, Cordes T, Ghelfi J, Pailot A, Reiling N, Goldmann O, et al. Immune-responsive gene 1 protein links metabolism to immunity by catalyzing itaconic acid production. *Proceedings of the National Academy of Sciences*. 2013;110(19):7820-5.
108. Mills EL, Kelly B, Logan A, Costa ASH, Varma M, Bryant CE, et al. Succinate dehydrogenase supports metabolic repurposing of mitochondria to drive inflammatory macrophages. *Cell*. 2016;167(2):457-70.
109. Pethe K, Sequeira PC, Agarwalla S, Rhee K, Kuhen K, Phong WY, et al. A chemical genetic screen in *Mycobacterium tuberculosis* identifies carbon-source-dependent growth inhibitors devoid of in vivo efficacy. *Nature communications*. 2010;1(1):1-8.
110. Singhal A, Jie L, Kumar P, Hong GS, Leow MK-S, Paleja B, et al. Metformin as adjunct antituberculosis therapy. *Science translational medicine*. 2014;6(263):263ra159-263ra159.
111. Ranjbar S, Haridas V, Nambu A, Jasenosky LD, Sadhukhan S, Ebert TS, et al. Cytoplasmic RNA sensor pathways and nitazoxanide broadly inhibit intracellular *Mycobacterium tuberculosis* growth. *Isience*. 2019;22:299-313.
112. Gutierrez MG, Master SS, Singh SB, Taylor GA, Colombo MI, Deretic V. Autophagy is a defense mechanism inhibiting BCG and *Mycobacterium tuberculosis* survival in infected macrophages. *Cell*. 2004;119(6):753-66.
113. Wallis RS, Hafner R. Advancing host-directed therapy for tuberculosis. *Nature Reviews Immunology*. 2015;15(4):255-63.
114. Khan N, Pahari S, Vidyarthi A, Aqdas M, Agrewala JN. Stimulation through CD40 and TLR-4 is an effective host directed therapy against *Mycobacterium tuberculosis*. *Frontiers in immunology*. 2016;7:386.
115. Bruns H, Stegelmann F, Fabri M, Döhner K, van Zandbergen G, Wagner M, et al. Abelson tyrosine kinase controls phagosomal acidification required for killing of *Mycobacterium tuberculosis* in human macrophages. *The Journal of Immunology*. 2012;189(8):4069-78.
116. Hu Y, Wen Z, Liu S, Cai Y, Guo J, Xu Y, et al. Ibrutinib suppresses intracellular *mycobacterium tuberculosis* growth by inducing macrophage autophagy. *Journal of Infection*. 2020;80(6):e19-e26.

117. Juárez E, Carranza C, Sánchez G, González M, Chávez J, Sarabia C, et al. Loperamide restricts intracellular growth of *Mycobacterium tuberculosis* in lung macrophages. *American journal of respiratory cell and molecular biology*. 2016;55(6):837-47.
118. Tsai IF, Kuo CP, Lin AB, Chien MN, Ho HT, Wei TY, et al. Potential effect of ezetimibe against *Mycobacterium tuberculosis* infection in type II diabetes. *Respirology*. 2017;22(3):559-66.
119. Parihar SP, Guler R, Khutlang R, Lang DM, Hurdal R, Mhlana MM, et al. Statin therapy reduces the *mycobacterium tuberculosis* burden in human macrophages and in mice by enhancing autophagy and phagosome maturation. *The Journal of infectious diseases*. 2014;209(5):754-63.
120. Vrieling F, Wilson L, Rensen PCN, Walzl G, Ottenhoff THM, Joosten SA. Oxidized low-density lipoprotein (oxLDL) supports *Mycobacterium tuberculosis* survival in macrophages by inducing lysosomal dysfunction. *PLoS pathogens*. 2019;15(4):e1007724.
121. Pahari S, Negi S, Aqdas M, Arnett E, Schlesinger LS, Agrewala JN. Induction of autophagy through CLEC4E in combination with TLR4: an innovative strategy to restrict the survival of *Mycobacterium tuberculosis*. *Autophagy*. 2020;16(6):1021-43.
122. Pajuelo D, Gonzalez-Juarbe N, Niederweis M. NAD hydrolysis by the tuberculosis necrotizing toxin induces lethal oxidative stress in macrophages. *Cellular microbiology*. 2020;22(1):e13115.
123. Moreira JD, Koch BEV, van Veen S, Walburg KV, Vrieling F, Mara Pinto Dabés Guimarães T, et al. Functional inhibition of host histone deacetylases (HDACs) enhances in vitro and in vivo anti-mycobacterial activity in human macrophages and in zebrafish. *Frontiers in immunology*. 2020;11:36.
124. Liu PT, Stenger S, Tang DH, Modlin RL. Cutting edge: vitamin D-mediated human antimicrobial activity against *Mycobacterium tuberculosis* is dependent on the induction of cathelicidin. *The journal of immunology*. 2007;179(4):2060-3.
125. Coussens AK, Wilkinson RJ, Martineau AR. Phenylbutyrate Is Bacteriostatic against *Mycobacterium tuberculosis* and Regulates the Macrophage Response to Infection, Synergistically with 25-Hydroxy-Vitamin D₃. *PLoS pathogens*. 2015;11(7):e1005007.
126. Rekha RS, Rao Muvva SSVJ, Wan M, Raqib R, Bergman P, Brighenti S, et al. Phenylbutyrate induces LL-37-dependent autophagy and intracellular killing of *Mycobacterium tuberculosis* in human macrophages. *Autophagy*. 2015;11(9):1688-99.
127. Coleman MM, Basdeo SA, Coleman AM, Cheallaigh CN, Peral de Castro C, McLaughlin AM, et al. All-trans retinoic acid augments autophagy during intracellular bacterial infection. *American journal of respiratory cell and molecular biology*. 2018;59(5):548-56.
128. Ouyang Q, Zhang K, Lin D, Feng CG, Cai Y, Chen X. Bazedoxifene suppresses intracellular *Mycobacterium tuberculosis* growth by enhancing autophagy. *Mosphere*. 2020;5(2):e00124-20.



Appendices

Nederlandse samenvatting

Dankwoord

Curriculum Vitae

List of Publications

Nederlandse Samenvatting

Tuberculose (TBC) is een infectieziekte die wereldwijd miljoenen levens eist en nog altijd een bedreiging vormt voor de volksgezondheid. TBC wordt veroorzaakt door de bacterie *Mycobacterium tuberculosis* (*Mtb*), en hoewel de meeste mensen die besmet raken niet ziek worden (latente TBC), kan de bacterie ernstige longinfectie veroorzaken (pulmonaire TBC) of verspreiden naar andere delen in het lichaam. Ondanks pogingen van de World Health Organization (WHO) om TBC wereldwijd onder controle te brengen, raken er ieder jaar 10 miljoen mensen besmet, waarvan een aanzienlijk deel ernstig ziek wordt of zelfs overlijdt. De COVID-19 pandemie heeft een grote impact gehad op de bestrijding van TBC en in 2020 kwamen er meer mensen te overlijden dan het jaar voorheen (1.5 miljoen versus 1.4 miljoen mensen), onder andere doordat mensen in ontwikkelingslanden beperkte toegang hadden tot diagnostiek en/of behandeling. Naar verwachting zal deze trend doorzetten in 2022, met een toename in mortaliteit tot gevolg.

De toename van het aantal *Mtb* stammen dat resistent is tegen de huidige antibiotica bemoeilijkt het onder controle brengen van TBC ernstig. Een andere uitdaging bij de bestrijding van TBC is de combinatie van TBC besmetting met type-2 diabetes: patiënten die lijden aan beiden ziekten hebben een lagere kans op genezing van TBC en een hogere kans op terugkerende infecties. Diabetes is een chronische ziekte waarbij het lichaam niet voldoende insuline aanmaakt (type I diabetes) of niet voldoende reageert op insuline productie (type II diabetes). Diabetes verdrievoudigt het risico om actieve TBC te ontwikkelen na infectie met de TBC-bacterie. De toename van antibiotica-resistentie en de opkomst van TBC onder diabetes patiënten zijn zorgwekkende ontwikkelingen die nieuwe en innovatieve behandelmethoden vereisen.

Een ander probleem bij de bestrijding van TBC is dat veel TBC patiënten geen tijdige diagnose – en dus behandeling – krijgen, waardoor ze ernstig ziek kunnen worden en kunnen bijdragen aan de transmissie van TBC. Huidige diagnostische methoden zoals microscopie en diagnostiek op basis van DNA (zoals PCR) zijn afhankelijk van sputum van de patiënt, maar in de praktijk is het lastig om dit slijm op te hoesten uit de diepe luchtwegen, wat kan resulteren in slechte kwaliteit samples en daardoor vals negatieve test uitslagen. Huidige sputum-onafhankelijke diagnostische methoden, zoals de bekende Mantoux test, of meer recente Quantiferon TBC bloed testen kunnen geen onderscheid maken tussen latente en actieve TB, waardoor de toepasbaarheid in TBC-endemische gebieden beperkt is. Genetische biomarkers kunnen een oplossing bieden voor bovengenoemde nadelen, omdat ze gemeten kunnen worden in het bloed en onderscheid kunnen maken tussen latente en actieve TBC. Biomarkers kunnen zowel gebruikt worden voor de diagnose van TBC als voor het monitoren van de respons van TBC patiënten op behandeling met medicijnen. Er is echter nog weinig bekend over de toepasbaarheid van

zulke biomarkers in patiënten die zowel TBC als diabetes hebben.

Diabetes interfereert met de genexpressie in TBC patiënten ten tijde van diagnose, zodat eerder gepubliceerde biomarker profielen van TBC patiënten zonder diabetes maar een gelimiteerde diagnostische waarde hebben in deze patiënten. In **hoofdstuk 2** hebben we nieuwe biomarker profielen geïdentificeerd, die zowel toepasbaar zijn in patiënten met ongecompliceerde TBC als in patiënten met TBC en diabetes. We hebben aangetoond dat patiënten met TBC en patiënten met TBC-diabetes aanzienlijke verschillen vertonen op het niveau van genexpressie tijdens de behandeling van TBC. Hiermee kunnen we meer inzicht krijgen in de immuunrespons van TBC-diabetes patiënten ten opzichte van TBC patiënten. Ondanks de verschillen, hebben we ook genen gekarakteriseerd die tijdens de behandeling van TBC gereguleerd worden in beide groepen patiënten, dus ongeacht diabetes en bloedsuiker status. Door middel van modellen hebben we aangetoond dat het stellen van de TBC diagnose op basis van deze gemeenschappelijke genen mogelijk is in patiënten met TBC en diabetes comorbiditeit. In **hoofdstuk 3** hebben we onderzocht of het mogelijk is om de klinische uitkomst van TBC-diabetes patiënten te voorspellen op basis van gen expressie profielen gemeten in het bloed. Helaas is de behandeling van TBC niet succesvol in ongeveer 15% procent van de TBC patiënten en dit percentage ligt zelfs nog hoger in TBC-diabetes patiënten, maar het is nog niet duidelijk welke factoren en genen hierbij een rol spelen. In dit hoofdstuk hebben we aangetoond welke gen expressie profielen tijdens behandeling van TBC gereguleerd worden in patiënten die genezen zijn na behandeling, ten opzichte van patiënten die niet genezen zijn na behandeling. Daarnaast hebben we door middel van ‘machine learning’ een model opgesteld dat op basis van het profiel van slechts 8 genen de klinische uitkomst van behandeling kan voorspellen, al voordat de behandeling gestart is. Daarnaast kon de klinische uitkomst ook twee weken na behandeling voorspeld worden op basis van het expressie profiel van 22 genen. Samen suggereren onze bevindingen dat ondanks grote verschillen tussen TBC patiënten en TBC-diabetes patiënten, het meten van genexpressie in bloed een essentiële bijdrage kan leveren aan de diagnostiek van TBC in diabetes patiënten.

Het tweede deel van dit proefschrift focust op de ontwikkeling van een nieuwe behandelmethode voor TBC. De immuunrespons van mensen die besmet zijn met de TBC-bacterie is bepalend voor de ontwikkeling van latente versus actieve TBC, waardoor het moduleren van de immuunrespons mogelijkheden biedt om de ziekte te voorkomen of te behandelen. We zijn daarom op zoek gegaan naar chemische stoffen die de immuunrespons moduleren, om de groei of overleving van de TBC-bacterie in menselijke cellen te remmen. Deze stoffen kunnen bijvoorbeeld binden aan menselijke eiwitten, de zogenaamde ‘targets’, om op deze manier een immuunrespons te induceren die nadelig is voor de bacterie. Het gebruik van dergelijke stoffen verkleint de kans op het ontstaan van

nieuwe resistentie vergeleken met traditionele antibiotica.

Vanwege het eerdergenoemde verband tussen TBC en diabetes, hebben we getest of targets die een beschreven rol spelen bij diabetes, belangrijk zijn tijdens TBC-infecties. In **hoofdstuk 4** hebben we ‘poly (ADP) ribose polymerases’ geïdentificeerd als humane targets. Remming van de activiteit van deze targets door middel van chemische stoffen resulteerde in verminderde aantallen van de TBC bacterie in humane macrofagen, de afweercellen die voornamelijk geïnfecteerd worden door de TBC-bacterie. In **hoofdstuk 5** hebben we onderzocht wat de rol van de stofwisseling van macrofagen is tijdens infecties met TBC en Salmonella, een andere pathogeen dat met name bekend staat als de veroorzaker van voedselvergiftigingen. Onze bevindingen suggereren dat het moduleren van de stofwisseling door middel van chemische stoffen zeer verschillende effecten had op deze twee bacteriën. Dit levert kennis op over de biologische interacties tussen de bacterie en humane cellen, waarmee we in de toekomst hopelijk potentiële stoffen kunnen vinden om deze bacteriën te remmen.

Ons werk levert handvatten op om TBC beter te kunnen bestrijden in patiënten met of zonder diabetes. We hebben ons gericht op twee strategieën die kunnen bijdragen aan de bestrijding van TBC, namelijk: (1) op TBC diagnostiek in diabetes patiënten en (2) op de ontdekking van chemische stoffen die aangrijpen op humane targets, die de immuunrespons van cellen tegen de TBC-bacterie verbeteren. Met deze kennis worden nieuwe klinische toepassingen mogelijk gemaakt om TBC beter te kunnen bestrijden.

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Curriculum Vitae

Cassandra Laura Rebecca van Doorn was born in Zwijndrecht, the Netherlands, on the 30th of April in 1993. After graduating from pre-university education in 2011, she started studying Biology at Leiden University. She participated in the minor 'Disease, Signaling and Drug Targets' of Bio-Pharmaceutical Sciences at Leiden University. In 2014, she performed her internship at the Department of Infectious Diseases of the Leiden University Medical Center (LUMC) under the supervision of dr. C.J. Korbé. During this internship she studied *in vitro* host interactions with the pathogen *Salmonella enterica*. After obtaining her Bachelor Degree in Biology in 2014, she started the Biology Master's program at Wageningen University with the specialization 'Health and Disease'. Her first master internship was performed at the department of Cell Biology and Immunology at Wageningen University. Under the supervision of dr. J. Petit, Cassandra optimized an *in vitro* long-term culture model of common carp macrophages to characterize innate immune memory. Her final master internship was performed at the department of Clinical Chemistry and Haematology at the University Medical Center Utrecht (UMCU). There, she developed an ELISA-based diagnostic tool to quantify cleaved high molecular weight kininogen production in human plasma as surrogate marker for bradykinin production under the supervision of dr. Z. Hofman. After obtaining her Master's Degree in 2016, she started her research to attain her Ph.D. at the Department of Infectious Diseases at the LUMC, resulting in this thesis.

List of Publications

1. Eckold C*, van Doorn CLR*, Ruslami R, Ronacher K, Costache A, van Veen S, Lee J, Kumar V, Kerry-Barnard S, Malherbe ST, Kleynhans L, Stanley K, Critchley JA, Hill PC, van Crevel R, Wijmenga C, Loana M, Alisjahbana B, Walzl G, Ottenhoff THM, Dockrell HM, Vianello E[#], Cliff JM[#], on behalf of the TANDEM Consortium (2022) Impaired resolution of blood transcriptomes through tuberculosis treatment with diabetes comorbidity. *MedRxiv*. 2022.02.07.22269422. doi: 10.1101/2022.02.07.22269422
2. van Doorn CLR*, Eckold C*, Ronacher K, Ruslami R, van Veen S, Lee J, Kumar V, Kerry-Barnard S, Malherbe ST, Kleynhans L, Stanley K, Hill PC, Joosten SA, van Crevel R, Wijmenga C, Critchley JA, Walzl G, Alisjahbana B, Dockrell HM, Ottenhoff THM, Vianello E[#] and Cliff JM[#], on behalf of the TANDEM Consortium (2022) Transcriptional profiles predict treatment outcome in patients with tuberculosis and diabetes at diagnosis and at two weeks after initiation of anti-tuberculosis treatment. *EBioMedicine*. 82:104173. doi: 10.1016/j.ebiom.2022.104173.
3. van Doorn CLR, Steenbergen SAM, Walburg KV and Ottenhoff THM (2021) Pharmacological Poly (ADP-Ribose) Polymerase Inhibitors Decrease *Mycobacterium tuberculosis* Survival in Human Macrophages. *Front. Immunol.* 12:712021. doi: 10.3389/fimmu.2021.712021
4. van Doorn CLR, Schouten GK, van Veen S, Walburg KV, Esselink JJ, Heemskerk MT, Vrieling F and Ottenhoff THM (2021) Pyruvate Dehydrogenase Kinase Inhibitor Dichloroacetate Improves Host Control of *Salmonella enterica* Serovar Typhimurium Infection in Human Macrophages. *Front. Immunol.* 12:739938. doi: 10.3389/fimmu.2021.739938

*These authors contributed equally

[#]These authors contributed equally