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& CELLULAR METABOLISM
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Host Response to Tuberculosis: Impact of Diabetes & Cellular Metabolism of Immune cells
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Impact of Diabetes & Cellular Metabolism
of Immune Cells

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Host Response to Tuberculosis:
Impact of Diabetes & Cellular Metabolism
of Immune Cells

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Chapter 1

Introduction, aim and outline of thesis
A HISTORICAL PERSPECTIVE

At the beginning of the 19th century, the French scientist Théophile Laennec systematically documented the physical and pulmonary signs of tuberculosis for the first time. He linked pulmonary lesions found on the lungs of autopsied patients with the symptoms in surviving tuberculosis patients. Following his work in 1865 Jean-Antoine Villemin demonstrated the transmissible nature of \textit{M. tuberculosis}. Villemin did this by infecting laboratory rabbits with tuberculous matter taken from an autopsy of an individual who had died of tuberculosis. The infected rabbits developed extensive tuberculosis three months after infection. Despite his findings, Villemin's work was ignored by the scientific community at that time. Nearly two decades later on 24 March in 1882 Robert Koch stumped his audience as he revealed that a bacterium, namely \textit{Mycobacterium tuberculosis}, was the etiologic agent of tuberculosis (Figure 1). During his lecture he used a microscope to show miniscule rod-like structures or bacilli that could cause tuberculosis in infected guinea pigs. His findings were made possible by the development of a new staining technique and a method for growing bacteria on slanted solid cultures. He received the Nobel Prize in physiology or medicine in 1905 for "his investigations and discoveries in relation to tuberculosis."
Chapter 1

Tuberculosis reached its peak in the 18th century when one in every four people died of tuberculosis in Great Britain. After that, various factors stemmed the spread of tuberculosis and reduced overall mortality rates, including Koch's identification of tuberculosis, the development of the tuberculin skin test, another legacy of Koch, and the understanding of the disease's contagious nature. In particular, social factors such as improved sanitation, the introduction of vaccination with bacille Calmette–Guérin (BCG) and heavy public health campaigns (Figure 2) all converged to reduce morbidity and mortality due to tuberculosis even before the arrival of antibiotics. Between 1946 and 1949, the development of the antibiotic streptomycin made effective treatment and cure of TB a reality. Today, the control and decrease in tuberculosis-related deaths is an advancement that is still clouded by the fact that tuberculosis remains a formidable disease in developing countries, killing nearly 1.8 million people in 2016 alone. Likewise, nearly one third of the world is a latent reservoir of tuberculosis. Thus 135 years after Robert Koch’s discovery we are still fighting the battle of tuberculosis.

Figure 2 | Public health posters used to reduce the spread of tuberculosis issued in the 1900s in the US, UK and France (from left to right). Copyright Details: Left (Courtesy of Library of Congress from where this image may be obtained at no cost https://www.loc.gov/item/98516354/), Middle (© IWM (Art.IWM PST 14135)) and Right (Retrieved from https://digital.library.illinois.edu/items/7e64fa40-0b13-0134-1d55-0050569601ca-2).
STAGES OF TUBERCULOSIS INFECTION

After aerosol-dependent infection, approximately 5% of infected individuals develop primary or active pulmonary tuberculosis within two years of infection either with or without a short period of latency\(^1\) (Figure 3). Development of active tuberculosis is most common in children suffering from certain primary immune-deficiencies\(^2\) or in adults suffering from HIV/AIDS\(^3\). However, most infected individuals develop latent TB, characterised by a positive diagnostic test such as the Mantoux test (TST) or interferon-gamma release assay (IGRA), but show no overt clinical symptoms\(^4\).

Another 5-10% of individuals with latent tuberculosis infection (LTBI) progress to active disease during their lifetime\(^5\). Factors that influence the progression from LTBI to active disease are thought to be reflective of an impairment in host defence. Genetic factors are believed to contribute, whilst co-morbidities that promote the transition from latent to active disease include the depletion of CD\(^4\)+ T cells during HIV infection\(^6\), smoking\(^7,8\), diabetes\(^9\) or the use of anti-TNF biological agents for treatment of rheumatoid arthritis (RA)\(^10\). Lastly, environmental factors such as age, malnutrition, the microbiome and low vitamin-D (VitD) levels\(^11\) may also affect susceptibility to varying degrees. Once disease is established, host driven inflammation, bacterial burden and drug resistance influence the success rate of antibiotic therapy.
Transmission of *M. tuberculosis* takes place by aerosols. Infection establishes latent tuberculosis in most people except for a small group of people known as early clearers, who manage to clear the mycobacteria through yet-to-be identified host defences. Progression to active tuberculosis depends on a multitude of factors including diabetes, smoking and HIV. Cure, death and transmissibility of the disease are affected by factors such as treatment availability, adherence to treatment and drug resistance. Data for annual deaths and active tuberculosis are as of 2015 published by the World Health Organisation (WHO). Images of people created by Gan Khoon Lay from the Noun Project.
PART I: Tuberculosis & Diabetes

Globally, 15% of tuberculosis cases are estimated to be attributable to diabetes$^{30}$. Systematic reviews have shown that individuals with diabetes are twice as likely to die during tuberculosis treatment$^{21}$, thrice as likely to develop active tuberculosis$^{18,22}$ and four times as likely to relapse following treatment$^{21}$. Figure 4A highlights the specific relative risks associated with TB-DM.

**Figure 4 | Diabetes increases the risk of active tuberculosis.** (A) Relative risk values associated with TB-DM patients compared to TB-only patients. Data obtained from Baker et al, in which 5 studies reported the risk of relapse, 23 reported deaths and 12 reported the combined outcome of failure and death. Relative risk value risk of TB in DM patients (13 studies) was taken from Jeon et al. (B) In red, number of people with active tuberculosis in 2015 (WHO statistics). In green, number of people with type 2 diabetes mellitus in 2014 (WHO statistics) at risk for active tuberculosis.

The number of people living with diabetes is predicted to rise from 415 million in 2015 to 642 million in 2040, amounting to a staggering 55% increase in diabetes prevalence$^{23}$. Figure 4B attempts to illustrate how diabetes stands to threaten the successful control of tuberculosis disease that we have thus far achieved. Importantly, the exact biological mechanisms underpinning the increase in susceptibility to tuberculosis are to date unclear.

Apart from tuberculosis, diabetes patients are susceptible to a wide range of infections. For instance, certain relatively rare infections occur almost exclusively in patients with diabetes, namely rhinocerebral mucormycosis$^{24,25}$, Fournier’s gangrene and *Klebsiella pneumoniae* liver abscess$^{26}$. Likewise, bacterial pneumonia$^{27}$, urinary tract infections (UTIs)$^{28}$, skin infections$^{29}$ and invasive fungal infections by *Candida* species$^{30,31}$ also occur more frequently in patients with diabetes. Several biological explanations for these infections exists. For instance, diabetic ketoacidosis$^{32,33}$ accounts for a large number of mucormycosis cases, glycosuria accounts for the increase in UTI infections and diabetic neuropathy, macroangiopathy and
microangiopathy account for the increased skin lesions, poor wound healing and extreme cases of gangrene observed in diabetes patients\textsuperscript{10}. Importantly, a general decrease in the functioning of cells of the immune system has also been widely reported in patients with diabetes\textsuperscript{31,34}. In the case of tuberculosis and diabetes, the question therefore is whether a particular trait of diabetes, akin to CD4\textsuperscript{+} T cell depletion in HIV patients, or whether a general decrease in immune defence mechanisms is the cause for increased susceptibility to tuberculosis.
PART II: Cellular metabolism of immune cells and host defense to tuberculosis

Immuno-metabolism is a nascent field of research aiming to understand the role that cellular metabolism has in controlling the responses of our immune system. The way that immune cells use energy substrates such as glucose, fatty acids or amino acids can determine the function of these cells, and in turn these pathways may be perturbed in several disease states, including diabetes mellitus.

During homeostasis, cells of the immune system transport glucose into the cytosol where it is metabolised by glycolysis into pyruvate, which is further oxidised in the mitochondria canonically by way of the tri-carboxylic cycle (TCA) and oxidative phosphorylation (OXPHOS) pathways\(^3\)\(^5\). During activation, such as stimulation or differentiation, reliance on these pathways for energy changes to accommodate the needs of the cell. In particular, the function, activation status, and nutrient availability dictates variations on these pathways to generate sufficient energy. A common example is that of LPS stimulation during which cells increase their reliance on glycolysis whilst decreasing OXPHOS based metabolism, a process known as the Warburg effect\(^3\)\(^6\),\(^7\).

Each cell type has a unique metabolic profile. Neutrophils predominantly rely on glucose and glycolysis for energy to fuel functions such as ROS production and NET formation\(^3\)\(^8\),\(^3\)\(^9\). Activated myeloid cells or lymphocytes metabolise glucose, glutamine and other substrates predominantly through glycolysis and the TCA cycle\(^3\)\(^5\). Alternatively, cell types with roles in surveillance and maintenance such as M2 macrophages or regulatory T cells rely more on oxidative pathways such as fatty acid oxidation and OXPHOS\(^4\)\(^0\),\(^4\)\(^1\).
Figure 5 | Metabolic Pathways that Influence Host Immune Responses. Immune cells need energy in the form of ATP to survive, grow, reproduce and perform specific functions. During homeostasis glucose (Glc) entering the cytosol is converted into pyruvate (Pyr) in a series of steps known as glycolysis. Pyruvate is then transported into the mitochondria where it is metabolised into CO\textsubscript{2} and 32 ATP via the tri-carboxylic (TCA) cycle and electron transport chain (ETC) in a process called oxidative phosphorylation (OXPHOS). Upon activation, glucose import into the cytosol is increased. This increases the glycolytic rates and thus availability of other glycolytic intermediates which serve as precursors for biosynthetic processes. The resulting excess pyruvate is converted into lactate (Lac) via aerobic glycolysis, which generates two molecules of ATP. Another product of glycolysis, glucose-6-phosphate (G6P), feeds into the pentose phosphate pathway (PPPW) which supports nucleotide biosynthesis and generates the co-factor NADPH. Pyruvate can also be converted to acetyl-CoA (Ac-CoA) which supports the synthesis of cholesterol and lipids, which in turn can be broken down by oxidation to re-enter the TCA cycle. Alternative fuels such as glutamine (Gln) or other amino acids enter into the tricarboxylic cycle and supply intermediates for biosynthesis. α-ketoglutarate (α-KG), Citrate (Cit), Glutamate (Glu), Malonyl – CoA (Ma-CoA), Oxaloacetate (OAA). © Ekta Lachmandas, inspired by Ghesquiere et al\textsuperscript{42}. 
Similar to the energy demands of different cell types, infection by pathogens modulate the metabolic landscape of immune cells. With regards to *M. tuberculosis*, studies in mice and isolated monocytes from humans have shown that mycobacteria induce glycolysis in immune cells and that inhibition of this pathway impairs mycobacterial clearance in human macrophages. Further investigation into the molecular mechanisms behind these and other described pathways will expand our repertoire of knowledge on the relation between tuberculosis and metabolism.

Last but not least, if metabolism is tightly linked with the immune response to tuberculosis, can cellular metabolism be clinically modulated to enhance anti-mycobacterial responses? This area of research is known as host-directed therapy and one such example would be that of metformin. Metformin is the most widely administered type II diabetes drug around the world. It functions by reducing blood glucose levels and improving insulin resistance. Metformin works through a multitude of mechanisms including mTOR inhibition, inhibition of complex I of the electron transport chain, and activation of AMPK. As a result, metformin profoundly influences various cellular immune functions. Metformin has already been proposed as adjunctive therapy for tuberculosis, but before confirming its suitability it must be thoroughly investigated in humans and controlled clinical studies.
AIM AND OUTLINE OF THIS THESIS

The previous paragraphs illustrate the susceptibility of patients with diabetes to tuberculosis and the role of metabolism in the immune response to tuberculosis. Both areas of research are in their infancy and hence many aspects of susceptibility and metabolism remain unknown. The aim of this thesis was to contribute to (1) uncovering the mechanisms behind the increased tuberculosis susceptibility in diabetes and (2) the investigation of changes in cellular metabolism that take place during tuberculosis infection.

PART I: The impact of diabetes on susceptibility to tuberculosis.

Hyperglycaemia is one of the hallmarks of type II diabetes and numerous studies have associated it with a compromised innate immune response. In Chapter 2 I examined if culture-controlled hyperglycaemia influences cytokine production, phagocytosis and the clearance of *M. tuberculosis* from infected human macrophages. Type II diabetes is a complex disease in which insulin resistance and dysregulated glucose metabolism associates with other metabolic traits such as raised cholesterol and triglyceride levels, hyperuricemia and hypertension. This mixed clinical picture makes it difficult to discern the specific influence of hyperglycaemia on tuberculosis in patients with type II diabetes. Therefore, in Chapter 3 we recruited a small cohort of type I diabetes patients with severely dysregulated hyperglycaemia and no other overt complications. Their cytokine and metabolic responses to tuberculosis were compared to matched healthy controls and the mechanisms that contributed to the observed differences were investigated.

The microbiota of people with diabetes is significantly altered compared to that of healthy individuals. Components of a healthy microbiota such as short chain fatty acids (SCFA) are known to modulate and control the immune response. In particular, butyrate-producing bacteria have been associated with a state of health. Butyrate is a small molecule that limits inflammation and thus disease associated pathology. To understand its role in tuberculosis, in Chapter 4 we examined the effects of SCFA and in particular butyrate on the immune response to tuberculosis and delineated the mechanisms behind their effects.
PART II: Cellular metabolism of immune cells and host defence to tuberculosis

The Warburg effect, a metabolic switch from glycolysis to OXPHOS, is the first metabolic change to have been described in an immunological context, specifically during stimulation with LPS. In Chapter 5 we investigated whether a similar switch to glycolysis takes place during tuberculosis. Specifically, transcriptional changes in glycolysis in patients with pulmonary tuberculosis were examined, validated and the molecular mechanisms behind them were elucidated.

When investigating the metabolic changes induced by mycobacteria, to our surprise we discovered that OXPHOS was not decreased but instead upregulated. This was in contrast to TLR4 stimulation by LPS. To understand the significance of these differences, monocytes were stimulated with a range of stimuli and the subsequent changes in OXPHOS metabolism were investigated. Chapter 6 covers this study using primarily LPS/TLR4 and P3C/TLR2 as model stimuli and links changes in metabolism to functions such as cytokine production and phagocytosis.

Having previously looked at individual pathways, in Chapter 7 we used a systems biology approach to identify global changes in metabolism in a TST skin model of tuberculosis infection. Genetic changes in a panel of relevant metabolic genes were analysed to determine if they could influence cytokine production to M. tuberculosis.

Metformin has been proposed as adjunctive therapy for tuberculosis and diabetes and a murine study that emerged during the course of this thesis described results to support this proposition. To evaluate the applicability of these studies in humans in Chapter 8 we examined the effect of metformin on several host defence mechanisms in healthy volunteers who were given metformin for five days.
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Introduction


PART I
The impact of diabetes on susceptibility to tuberculosis
Chapter 2

The effect of hyperglycaemia on in vitro cytokine production and macrophage infection with *Mycobacterium tuberculosis*

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ABSTRACT

Type 2 diabetes mellitus is an established risk factor for tuberculosis but the underlying mechanisms are largely unknown. We examined the effects of hyperglycaemia, a hallmark of diabetes, on the cytokine response to and macrophage infection with *Mycobacterium tuberculosis*. Increasing *in vitro* glucose concentrations from 5 to 25 mmol/L had marginal effects on cytokine production following stimulation of peripheral blood mononuclear cells (PBMCs) with *M. tuberculosis* lysate, LPS or *Candida albicans*, while 40 mmol/L glucose increased production of TNF-α, IL-1β, IL-6 and IL-10, but not of IFN-γ, IL-17A and IL-22. Macrophage differentiation under hyperglycaemic conditions of 25 mmol/L glucose was also associated with increased cytokine production upon stimulation with *M. tuberculosis* lysate and LPS but in infection experiments no differences in *M. tuberculosis* killing or outgrowth was observed. The phagocytic capacity of these hyperglycaemic macrophages also remained unaltered. The fact that only very high glucose concentrations were able to significantly influence cytokine production by macrophages suggests that hyperglycaemia alone cannot fully explain the increased susceptibility of diabetes mellitus patients to tuberculosis.
INTRODUCTION

Type 2 diabetes mellitus (DM) has been increasingly recognized as an important risk factor for tuberculosis (TB). Epidemiological studies have demonstrated that adults with diabetes have a significantly increased risk of developing active TB [1] and it is estimated that globally 15% of TB cases are attributable to DM [2]. The global prevalence of DM will rise by an estimated 55% over the next 20 years, with the largest increases in TB endemic regions of Africa and Asia [3]. As a result, DM will become an increasingly important factor contributing to the sustained TB epidemic [4,5].

The causative pathogen of TB, *Mycobacterium tuberculosis* (MTB), primarily infects phagocytic cells of the lung, such as alveolar macrophages. The early stage of infection is characterised by the recruitment and accumulation of various innate immune cells at the site of infection including neutrophils, dendritic cells and interstitial macrophages, the latter of which subsequently become infected by the growing population of mycobacteria and ultimately develop into bacteria-sequestering granulomas [6,7]. Effective immunity against MTB is dependent on the production of pro-inflammatory cytokines like interferon-γ (IFN-γ) and tumour necrosis factor-α (TNF-α) [8–11], whilst anti-inflammatory cytokines such as interleukin-(IL)-10 can attenuate the anti-bacterial immune response [10,12].

It has been hypothesised that alterations in the immune response of patients with diabetes give rise to either an enhanced susceptibility to infection or accelerated progression towards active TB disease [5]. A possible explanation for these immunological changes is chronic hyperglycaemia, a hallmark of DM. Various studies have demonstrated that diabetes and hyperglycaemia in particular is associated with a compromised innate immune response which includes impairments in phagocytosis, cytokine secretion and macrophage activation [13–18]. Other studies that have investigated the adaptive arm of the immune response have yielded conflicting results when comparing differences in cytokine production between diabetes patients with or without TB [19–21]. These inconsistent data illustrate both the complexity of the interaction between TB and DM and reveal limitations in the comparability of studies that use divergent methods, such as differences in cellular origins and patient populations.

To better understand the effects of hyperglycaemia on the innate immune response during concurrent diabetes and tuberculosis, we investigated whether elevated concentrations of glucose could directly regulate the functional capacities of human macrophages *in vitro*. We initially determined the effects of hyperglycaemia on the cytokine response of PBMCs and macrophages after stimulation with bacterial lipopolysaccharide (LPS) or whole pathogen...
lysates, and later in alternatively activated (M2) macrophages upon infection with the MTB strain H37Rv. Finally, we assessed the phagocytic ability of hyperglycaemic M2 macrophages and studied their capacity to support mycobacterial outgrowth.
METHODS

Ethics Statement
PBMCs were isolated from buffy coats donated after written informed consent by healthy volunteers to the Sanquin Bloodbank (http://www.sanquin.nl/en/) in Nijmegen and Leiden. Blood was collected anonymously which, according to institutional ethical policy, does not require a separate review by the Ethical Committee. Experiments were conducted according to the principles expressed in the Declaration of Helsinki.

Healthy Volunteers
Since blood donations were anonymous no tuberculosis skin test or IFN-γ release assay could be performed but the incidence of TB in the indigenous Dutch population is extremely low (4/100,000) and Bacillus Calmette-Guérin (BCG) vaccination is not part of the routine vaccination program. The incidence of diabetes mellitus in the Dutch population for persons under 25 years of age is less than 1% and under 45 years of age is about 1.5%. Since the average age of blood donors in these experiments is approximately 45 years [22] we expect that almost none of them would have diabetes mellitus which would otherwise act as a confounding factor [23].

Cytokine Stimulation Experiments
Isolation of peripheral blood mononuclear cells (PBMCs) was performed by differential centrifugation over Ficoll-Paque (GE Healthcare) within 0-2 hours of collection. After counting (Casy Counter) cells were adjusted to $5 \times 10^6$ cells/mL were suspended in glucose free RPMI 1640 (Gibco) supplemented with 50 mg/mL gentamicin (Lonza) and 2 mM L-glutamine (Life Technologies). 100 μL of PBMCs was incubated in flat bottom 96-well plates (Greiner) with varying final glucose concentrations from 5 mmol/L to 40 mmol/L D-glucose (Sigma-Aldrich) in glucose-free RPMI, and stimulated with 1 μg/mL of H37Rv lysate, $1 \times 10^6$ microorganisms/mL of heat-killed Candida albicans (ATCC MYA-3573 (UC 820)) or 10 ng/mL LPS (Sigma-Aldrich, E. coli serotype 055:B5). The plates were incubated for 24 h, 48 h or 7 days at 37°C in a 5% CO₂ environment. Alternatively, 100 μL of PBMCs ($5 \times 10^6$/mL) was incubated for 1 h at 37°C in 5% CO₂ and adherent monocytes were selected by washing out non-adherent cells with warm PBS. Adherent monocytes were differentiated into M2 macrophages (n=18) in 10% human pooled serum and 50 ng/mL M-CSF (R&D Systems) or M0 macrophages (n=23) in 10% human pooled serum for 6 days. Monocytes were differentiated in the presence of 5 mmol/L or 25 mmol/L glucose. In some cases monocytes were differentiated in 5 mmol/L glucose with 20 mmol/L mannitol (Sigma-Aldrich) to control for effects of osmolarity on cytokine production. Media containing M-CSF and/or
Chapter 2

serum and glucose were refreshed on day 3 of differentiation. Differentiated macrophages were stimulated on day 6 with RPMI (negative control), 1 μg/mL H37Rv lysate or 10 ng/mL LPS (positive control). Cell culture supernatants were collected after 24 h.

**Cytokine Measurements**

Cell culture supernatants were collected and stored at -20°C for cytokine measurements, which were performed by ELISA: IL-1β, TNF-α, IL-17A, IL-22 (R&D Systems); IL-6, IFN-γ and IL-10 (Sanquin).

**H37Rv Lysates and Culture**

Cultures of H37Rv MTB were grown to mid-log phase in Middlebrook 7H9 liquid medium (Difco, Becton-Dickinson) supplemented with oleic acid/albumin/dextrose/catalase (OADC) (BBL, Becton-Dickinson), washed three times in sterile saline, heat killed and then disrupted using a bead beater, after which the concentration was measured using a bicinchoninic acid (BCA) assay (Pierce, Thermo Scientific).

**H37Rv Infection of M2 macrophages**

CD14+ monocytes were isolated from PBMCs by magnetic cell sorting with anti-CD14-coated beads (Miltenyi Biotec) and seeded in tissue culture-treated flasks (Corning). After 6 days of differentiation in the presence of 50 ng/mL M-CSF, M2 macrophages were harvested using trypsin and transferred to tissue culture-treated 24-well plates (Corning) with 300,000 macrophages per well. Macrophages were incubated O/N at 37°C in 5% CO2 and subsequently infected with the H37Rv strain of MTB. Mycobacterial cultures were diluted to pre-log phase density one day before infection to ensure that the bacteria were in the log phase of the growth curve. Bacterial density was determined by measuring optical density at 600 nm (OD-600) and the bacterial suspension was diluted to a concentration of 30 x 10^6 bacteria/mL (MOI 10:1). 100 µL of the bacterial suspension was added to the cell cultures, after which the plates were centrifuged for 3 minutes at 800 rpm and incubated at 37°C in 5% CO₂. After 60 minutes the plates were washed with culture medium containing 30 µg/mL gentamicin and subsequently incubated O/N at 37°C in 5% CO₂ in medium containing 5 µg/mL gentamicin. Supernatants were collected and filtered before cytokine measurements. M2 macrophages were lysed in water for 5 minutes and plated on Middlebrook 7H10 agar (Difco, Becton-Dickinson) supplemented with OADC. CFUs were determined after 2-3 weeks.
Cell viability assay

Macrophage viability during prolonged H37Rv infection was assessed by using a LDH cytotoxicity kit according to the manufacturer’s instructions (Pierce, Thermo Scientific). For each experimental condition 50 µL of freshly harvested supernatant was incubated with 50 µL LDH reaction mixture for 30 minutes at room temperature in a 96-well plate. The reaction was stopped by adding 50 µL of stop solution and the plate was subsequently measured on a Mithras LB 940 microplate reader (Berthold Technologies) at 485 nm. Spontaneous and maximum LDH release controls were included in order to calculate the percentage of cytotoxicity. Macrophages were additionally stained with Trypan Blue (Sigma-Aldrich) as a second measure of cell viability.

Phagocytosis Quantification Assay

To quantify phagocytic capacity, fluorescent polystyrene particles (Fluoresbrite YG carboxylate microspheres) were used as described by Leclerc et al [24]. In short, M2 macrophages were incubated with P-beads in a ratio of 10 beads to 1 cell for 90 minutes at 37°C. Subsequently cells were collected with a cell scraper and centrifuged at 1500 rpm for 10 minutes. Following centrifugation supernatant was discarded and cells were re-suspended in 100 µL culture medium or 100 µL culture medium and Trypan Blue (1:1) (Sigma-Aldrich). Internalisation of the beads was quantified by flow cytometry. Non-internalised beads emitted a red fluorescent signal after Trypan Blue quenching which was detected in the FL-3 channel whereas internalised beads were detected in the FL-1 channel.

Statistical Analysis

Differences were analysed using a Wilcoxon signed rank test (paired, non-parametric analysis) unless otherwise stated. Data was considered statistically significant at a p-value <0.05. Data are shown as cumulative results of levels obtained in all volunteers (means ± SEM).
RESULTS

Hyperglycaemic culture conditions variably affect cytokine production from PBMCs. Little or no difference was seen in cytokine production following stimulation and culture of PBMCs in glucose concentrations ranging from 5 to 25 mmol/L, while culture in 40 mmol/L glucose mostly led to higher cytokine production (Figure 1). Production of pro-inflammatory cytokines IL-6 and IL-1β significantly increased in a dose dependent manner upon H37Rv stimulation whereas TNF-α production only increased at the highest glucose concentrations of 40 mmol/L. Production of the anti-inflammatory cytokine IL-10 also increased upon H37Rv lysate and Candida stimulations in the presence of 25 mmol/L or 40 mmol/L glucose. In comparison, induction of T-cell cytokines showed a high degree of variability in response to varying glucose concentrations. Overall, compared to normal glucose concentrations of 5 mmol/L, 40 mmol/L glucose showed the most significant changes in cytokine production (all p-values <0.05).

Macrophage differentiation in hyperglycaemic conditions leads to hyper-responsive cytokine production. The effect of hyperglycaemia on PBMC cytokine production was mild and mainly observed at the highest glucose concentrations (40 mmol/L). Both 25 mmol/L and 40 mmol/L of glucose can be observed in diabetes patients although the latter is rarely seen and is thus unlikely to account for the increase in TB susceptibility. We therefore proceeded by differentiating monocytes into M0 (serum derived) and M2 (M-CSF and serum derived) macrophages in the presence of 5 mmol/L glucose or the more clinically relevant hyperglycaemic condition of 25 mmol/L glucose. M2 macrophage differentiation was verified by assessing cell surface marker expression (CD14+/CD163+; Supplementary Figure 1). Differentiated macrophages were stimulated with LPS or H37Rv lysate (Figure 2A). Cytokine production was generally higher in macrophages differentiated under high glucose concentrations, although not all differences were statistically significant. Pro-inflammatory cytokines (TNF-α and IL-6) and anti-inflammatory cytokines (IL-10 and IL-1RA) from hyperglycaemic M2 macrophages were significantly increased after stimulation with H37Rv lysate and LPS. M0 macrophages displayed a similar pattern, except for TNF-α production.
Figure 1 | Cytokine production by PBMCs in response to antigenic stimuli in varying concentrations of glucose. (A) PBMCs were stimulated in the presence of 5 mmol/L to 40 mmol/L glucose with RPMI, H37Rv lysate (1 μg/mL), LPS (10 ng/mL) or heat-killed Candida Albicans (1 × 10^6 microorganisms/mL). Cell culture supernatants were collected after 24 h, 48 h or 7 days. (B) Data are shown as mean ± SEM of supernatant TNF-α, IL-1β, IL-6, IFN-γ, IL-17, IL-22 and IL-10 levels obtained in 6 volunteers, *p<0.05, **p<0.01.
Figure 2 | Cytokine production by M0 and M2 macrophages stimulated in varying concentrations of glucose. (A) Adherent monocytes were differentiated in either 5 or 25 mmol/L glucose into M0 macrophages (serum only) or M2 macrophages (M-CSF and serum) for 6 days and stimulated with RPMI, H37Rv lysate (1 μg/mL) or LPS (10 ng/mL). (B) Cell culture supernatants were collected and the pro-inflammatory cytokines TNF-α and IL-6 were measured along with the anti-inflammatory cytokines IL-10 and IL-1RA (n = 23). Data are shown as mean ± SEM, *p<0.05, **p<0.01 & ***p<0.001.
Figure 3 | Effects of euglycaemic and hyperglycaemic culture conditions on H37Rv infection and cytokine production in vitro. (A) CD14+ selected monocytes were differentiated into M2 macrophages in the presence of 5 mmol/L glucose, 5 mmol/L glucose and 20 mmol/L mannitol or 25 mmol/L glucose. The macrophages were infected for 1 hour with H37Rv at an MOI of 10. After infection the macrophages were washed and fresh media containing the different glucose media was added. After 24 hours supernatants were collected and the cells were lysed by osmotic pressure. (B) Cell lysates were serially diluted and plated on Middlebrook 7H10 agar. CFUs were counted after 2–3 weeks of growth at 37°C. Data are shown as mean ± SEM of two independent experiments (n = 4). (C) Pro-inflammatory cytokines TNF-α and IL-6 were measured along with the anti-inflammatory cytokines IL-10 and IL-1RA. Data are shown as mean ± SEM.
Sub-maximal concentrations of LPS and H37Rv were used to provide room for the potential boosting effects of hyperglycaemia on cytokine production.

Given the nature of these experiments the observed increase in cytokine production may have simply been a consequence of increased osmolarity during cell culture. To control for this, mannitol was used to achieve similar osmolarity in the 5 mmol/L and 25 mmol/L culture conditions (Supplementary Figure 2). In most cases the elevated cytokine production from M0 and M2 hyperglycaemic macrophages, as seen in Figure 2B, was reproducible under osmolarity controlled conditions. Thus the effects of hyperglycaemia on cytokine production cannot simply be explained by a difference in osmolarity.

Hyperglycaemia does not affect the cytokine response to and the survival of \textit{M. tuberculosis} in human macrophages. After investigating the cytokine profiles of macrophages stimulated with either LPS or H37Rv lysate in the presence of varying glucose concentrations the effect of hyperglycaemia on the \textit{in vitro} infection of M2 macrophages with the H37Rv strain of MTB was determined. M2 macrophages were used for the infection experiments as we have previously shown that this macrophage subtype is more adept in supporting mycobacterial survival and could therefore serve as the primary bacterial reservoir in the lungs during MTB infection [25]. After 24 h of infection no differences in CFU counts (Figure 3B) or cytokine production (Figure 3C) were observed between euglycaemic (5 mmol/L) and hyperglycaemic (25 mmol/L) macrophages. We also examined the effect of hyperglycaemia on the phagocytic capacity of macrophages and outgrowth of H37Rv after prolonged infection. M2 macrophages were able to control MTB growth as was demonstrated by a 1 log reduction in CFU over time (Figure 4B). However, differentiation and stimulation of M2 macrophages in the presence of 5 or 25 mmol/L of glucose were not associated with differences in the phagocytosis of fluorescent P-beads (Figure 4A), mycobacterial uptake (D0 Sups) and H37Rv survival throughout the course of infection (Figure 4B). We assessed macrophage viability during infection by measuring LDH release and staining the cells with Trypan Blue and found no differences in viability between glucose conditions (Supplementary Figure 3). Together these data demonstrate that hyperglycaemia influences neither \textit{in vitro} H37Rv infection and survival nor the infection-induced cytokine response in human M2 macrophages.
Figure 4 | Effects of high glucose on phagocytic capacity of macrophages and infection with H37Rv. M2 macrophages differentiated in the presence of 5 or 25 mmol/L glucose were either incubated with P-beads and subjected to flow cytometry measurements to determine the percentage of phagocytic cells (A) or were infected for 1 hour with H37Rv at an MOI of 10:1 (B). After infection macrophages were washed three times and fresh RPMI containing the different glucose media was added. The first wash (Day 0 Sups) of each infection was collected and plated in serial dilutions to determine whether different amounts of bacilli were taken up by euglycaemic or hyperglycaemic macrophages. Simultaneously macrophages (D0) were lysed and plated for CFU counts. Infected macrophages were also lysed on Day 1 (D1), Day 3 (D3), Day 5 (D5) and Day 7 (D7) after infection. CFUs were counted at once after 2–3 weeks of growth at 37°C. Data are shown as mean ± SEM (n = 4).
DISCUSSION

Type 2 diabetes mellitus confers a three-fold increased risk for active tuberculosis, but the underlying immunological mechanisms have not been identified [1]. In this study we investigated the effects of hyperglycaemia on in vitro cytokine production and mycobacterial infection. Hyperglycaemia altered cytokine production by PBMCs and macrophages stimulated with H37Rv lysate, although significant effects were mainly observed at the higher end of the glucose concentration range. Alternatively, hyperglycaemia did not affect the phagocytic capacity of macrophages or their ability to control outgrowth of H37Rv over a period of time.

PBMCs incubated in high glucose concentrations produced higher levels of TNF-α, IL-1β and IL-6, whilst IFN-γ, IL-17A and IL-22 levels did not change. This suggests that hyperglycaemia mainly affects monocytes but not T cells. As a result, we investigated whether hyperglycaemia had a more specific effect on macrophage-derived cytokine production. An increase in IL-6, IL-10 and IL1RA levels was found when hyperglycaemic monocyte-derived macrophages were stimulated with MTB and LPS.

To our knowledge, no studies have presented data on the effects of high glucose levels on in vitro cytokine production in response to MTB. Previous studies that examined ex vivo cytokine production in diabetes patients with or without TB have provided conflicting results. Some studies have shown elevated production of pro-inflammatory cytokines from whole blood of patients with TB-DM whereas another study using whole blood and one using PBMCs reported defects in IFN-γ production in patients with TB-DM [19–21,26]. Interestingly, both the increase and decrease of pro-inflammatory cytokines were correlated to increased HbA1c levels [19,26]. Differences between studies can be explained by the use of different cell types and stimuli. In this study we chose to investigate M0 (serum derived) and M2 (M-CSF and serum derived) macrophages, as they most closely represent tissue resident macrophages such as alveolar macrophages, in which MTB dominantly resides. Furthermore, patient studies are often complicated by variations in age, HBA1c levels, metabolic perturbations, medication etc., making it difficult to specifically examine the effects of hyperglycaemia. For these reasons we chose to exclusively study the effects of hyperglycaemia in vitro.

In contrast to the effects on cytokine production, the capacity of macrophages to phagocytose P-beads remained unaltered under high glucose concentrations. In literature, several studies report findings that both support and contrast with our observations on the effects of hyperglycaemia or DM on the phagocytic capacity of macrophages. In a TB-DM animal model in particular no significant differences were found in the phagocytic
capacity of alveolar macrophages from diabetic and non-diabetic rats subjected to aerosol infection with MTB [27,28]. In patients with DM the phagocytic function of macrophages and polymorphonuclear cells (PMN) is even more unclear [14,29–31]. In a recent study comparing patients with pulmonary TB, DM or the combination of TB and DM, no differences were found in the ability of PMNs to phagocytose, produce hydrogen peroxide or reduce nitroblue tetrazolium. In contrast, two studies from the same group using monocytes from patients with diabetes [32] showed a reduced association of MTB bacilli and reduced phagocytosis via the complement or Fc-γ receptor pathway, although this was not demonstrated in the context of MTB itself [33].

Similar to phagocytosis, no differences were found in MTB killing or outgrowth between hyperglycaemic and euglycaemic macrophages. To our knowledge no other data have been published on outgrowth of MTB in hyperglycaemic macrophages or macrophages from patients with DM. Of interest however is one study showing increased tuberculosis susceptibility in mice with streptozotocin-induced diabetes. In line with our study no differences in CFU counts were found in the lungs of acute diabetic mice. These results may indicate that hyperglycaemia may have long-term effects on susceptibility that are difficult to emulate in vitro [27,34].

Several aspects and limitations of our studies should be considered when discussing the relatively mild effects of hyperglycaemia on the immune responses elicited by MTB. Firstly, although hyperglycaemia did not directly affect MTB survival in macrophages, it is possible that it does after longer periods of time, or contributes to increased susceptibility to infection indirectly through effects on other immune cells. Secondly, even though hyperglycaemia is regarded as a major hallmark of DM, the pathophysiology of the disease is not restricted to high glucose concentrations. Other physiological disturbances in DM such as hyperinsulinaemia, diabetic acidosis and metabolic changes have also been found to affect immune cell functions, [35–37] and studies to assess their effects on MTB-induced immune responses are needed. Thirdly, DM is often associated with diet-induced conditions like dyslipidaemia. As MTB has been found to modulate host lipogenic pathways to survive in macrophages [38] it is possible that changes in blood lipid levels or composition contribute to the increased risk of active TB disease in DM patients. Furthermore we cannot exclude that the unidentified donors used in these experiments suffered from co-morbidities such as diabetes, although the chance of that are <1.5% as described above. Finally, it is unclear how accurately our in vitro model of hyperglycaemia reflects the in vivo situation during DM.
In short, these *in vitro* studies in PBMC and macrophages suggest that hyperglycaemia cannot fully explain the increased susceptibility to MTB in DM patients. Further studies that explore a broader range of metabolic parameters and cell types are needed to unravel the precise mechanisms underlying the effect of DM on TB.
In-vitro hyperglycaemia

In short, these in vitro studies in PBMC and macrophages suggest that hyperglycaemia cannot fully explain the increased susceptibility to MTB in DM patients. Further studies that explore a broader range of metabolic parameters and cell types are needed to unravel the precise mechanisms underlying the effect of DM on TB.

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Supplementary Figure 1 | M2 macrophage surface marker expression. M2 macrophage differentiation was verified by analysing the cell surface expression of CD14 (FITC, clone HCD14) and CD163 (Alexa 647, clone RM3/1) by flow cytometry. The FACS plots display representative results for CD14+/CD163+ M2 macrophages (black) versus an unstained sample (grey).
Supplementary Figure 2 | Effects of osmolarity on cytokine production from differentiated macrophages. Monocytes were differentiated into M0 or M2 macrophages in the presence of 5 mmol/L glucose, 5 mmol/L glucose and 20 mmol/L mannitol, or 25 mmol/L glucose, and subsequently stimulated with RPMI, H37Rv lysate (1 μg/mL) or LPS (10 ng/mL). Cell culture supernatants were collected after 24 h and the pro-inflammatory cytokines TNF-α and IL-6 were measured along with the anti-inflammatory cytokines IL-10 and IL-1RA (n=6). Data are shown as mean ± SEM, *p<0.05, **p<0.01 and ***p<0.001.
Supplementary Figure 3 | M2 macrophage viability during prolonged H37Rv infection. M2 macrophages differentiated for 6 days in the presence of 5 or 25 mmol/L glucose were infected for 1 hour with H37Rv at an MOI of 10:1. After infection macrophages were washed three times and fresh RPMI containing the different glucose media was added. The percentage of cytotoxicity was assessed by measuring LDH release from day 0 to day 7 corrected using spontaneous and maximum LDH release controls per time point. Macrophages were additionally stained with Trypan Blue as a second measure of cell viability and the resulting percentage of viable cells is indicated in the graph with an * at each time point for both conditions. Data are shown as mean ± SD (n=2).
Chapter 3

Patients with type 1 diabetes mellitus have impaired IL-1β production in response to *Mycobacterium tuberculosis*
ABSTRACT

Purpose: Patients with diabetes mellitus have an increased risk of developing tuberculosis. Although the underlying mechanism is unclear evidence suggests a role for chronic hyperglycemia.

Methods: We examined the influence of hyperglycaemia on Mycobacterium tuberculosis (M. tuberculosis) induced cytokine responses in patients with type 1 diabetes mellitus (T1D). PBMCs from 24 male T1D patients with suboptimal glucose control (HbA1c > 7.0% (53mmol/l)) and from 24 age-matched male healthy controls were stimulated with M. tuberculosis lysate. Cytokine analysis, assessment of aerobic glycolysis, receptor recognition and serum cross-over experiments were performed to explore the mechanistic differences.

Results: PBMCs from T1D patients produced less bioactive IL-1β in response to M. tuberculosis. IL-6 and IFN-γ production trended towards a decrease, whilst other cytokines such as TNF-α, IL-17 and IL-1ra were normal. The decrease in cytokine production was not correlated to HbA1c or plasma glucose levels. Cross-over serum experiments did not alter the cytokine profile of T1D or control patients arguing for an intrinsic cellular defect. Cellular metabolism and the expression of M. tuberculosis related pattern recognition receptors such as TLR2, TLR4 and NOD2 did not differ between T1D patients and healthy controls.

Conclusions: Compared to matched controls T1D patients have a reduced capacity to produce pro-inflammatory cytokines in response to M. tuberculosis, which may contribute to the increased susceptibility to tuberculosis. This effect appears not to be related to prevailing glucose levels but to an intrinsic cellular deficit.
INTRODUCTION

Diabetes increases the risk of developing active tuberculosis (TB) and is associated with worsened outcomes during TB treatment. It has been estimated that 15% of TB cases globally can be attributed to diabetes\(^1\). Patients with type 1 diabetes (T1D) may have an even higher risk of developing TB compared to those with type 2 diabetes (T2D)\(^2, 3, 4\). Additionally, poor glucose control escalates the risk of TB\(^5, 6\). The epidemiological evidence for the relation between diabetes and TB is strong, but the molecular and immunological basis for the susceptibility to TB remains largely unclear.

Recent evidence that points towards a disturbed innate and adaptive immune response to TB is mainly derived from studies in T2D patients\(^7\). However, T2D is a multifactorial disease involving age, obesity, sedentary lifestyle and genetics. It is characterised by hyperglycaemia, insulin resistance, hypertension, dyslipidemia and oxidative stress. All these factors, including the use of antidiabetic drugs with potential immune modulating capacities (i.e. metformin), make it difficult to specifically examine the role of hyperglycemia in the increased susceptibility to TB.

Therefore, we sought to study the response to \textit{M. tuberculosis} in T1D patients with chronic hyperglycaemia. We excluded individuals using drugs other than insulin replacement therapy and also those with serious diabetic complications. We examined whether T1D is associated with altered production of pro-inflammatory cytokines such as TNF, interleukin (IL)-1\(\beta\) and IL-6 from monocytes and interferon-\(\gamma\) (IFN-\(\gamma\)) from CD4\(^+\) lymphocytes, all of which are pivotal for effective host defenses against tuberculosis\(^8\). Finally, we examined whether external factors in serum (i.e. hyperglycaemia) or intrinsic factors (i.e. cellular metabolism and expression of pattern recognition receptors) were responsible for differences in cytokine responses between healthy controls and T1D patients.
METHODS

Recruitment and characterization of study subjects
We enrolled 24 male T1D patients with an HbA1c > 7.0% (53 mmol/l) and 24 age-matched male healthy controls. Participants were all between 20 and 70 years old. For T1D patients, the minimal duration of diabetes was 1 year. Patients using medication other than insulin were excluded. HbA1c was measured by standard laboratory methods. Plasma insulin was measured by radioimmunoassay. Plasma cholesterol, TG, glucose (Liquicolor, Human GmbH) and free fatty acids (NEFA-C WAKO chemicals, GmbH) were measured enzymatically following manufacturer’s protocols. Blood was drawn from a cubital vein and collected into sterile EDTA tubes for isolation of PBMCs or from serum tubes (Monoject). The study was approved by the Institutional Review board and written informed consent was obtained from all subjects. Using similar criteria an additional 6 T1D and 6 controls were recruited for a follow-up experiment.

PBMC isolation and stimulation
PBMC isolation was performed by dilution of blood in pyrogen-free PBS and differential density centrifugation over Ficoll-Paque (GE healthcare, UK). Cells were washed twice in PBS and re-suspended in RPMI culture medium (Roswell Park Memorial Institute medium; Invitrogen, CA, USA) supplemented with 5 mM glucose, 10 μg/ml gentamicin, 10 mM L-glutamine, and 10 mM pyruvate. PBMCs were counted with a Coulter counter (Coulter Electronics) and adjusted to 5×10⁶ cells/ml. A 100 μl volume was added to round bottom 96-well plates (Greiner, Frickenhausen, Germany) for PBMC stimulation experiments. Excess unstimulated PBMCs were lysed in TRIzol reagent (Invitrogen) and stored at −80°C until RNA isolation was performed.

Cells were stimulated with RPMI, 1 μg/ml Mycobacterium tuberculosis lysate for 24 h or 7 d (in the presence of 10% human pool serum for lymphocyte derived cytokines). For serum cross-over experiments, cells were incubated with 25% serum for 24 h or 7 d. In a follow-up experiment, we collected six additional male T1D patients with an HbA1c > 7.0% and six age-matched male healthy controls. PBMCs were isolated and stimulated with Escherichia coli LPS (serotype 055:B5, Sigma-Aldrich, 1 ng/mL or 10 ng/ml). Supernatants were collected and stored at -20°C until cytokine/lactate measurements were performed.
Cytokine measurements
Cytokine measurements from cell culture supernatants were performed by ELISA; namely interleukin (IL)-1β, IL-1 receptor antagonist (IL-1Ra), tumour necrosis factor (TNF)-α (R&D) and IL-6 (Sanquin) were measured in the 24 h PBMC stimulation experiments. Supernatants of the 7 d stimulations were used to measure IL-22, IL-17 (R&D Systems) or IFN-γ (Sanquin).

Bioactive IL-1 assay
Active IL-1 was measured indirectly using the mouse thymoma EL4-NOB1 (NOB1) cell line. NOB1 cells were cultured in RPMI culture medium supplemented with 1 mM pyruvate, 1 mM glutamax, 1 mM penicillin/streptomycin and 10% foetal bovine serum (Gibco) until confluence was reached. NOB1 cells (10^5 cells/well) were plated in a flat bottom 96-wells plate (Greiner). 70 µL (2x dilution) of supernatant from PBMCs of T1D or healthy controls that were stimulated with M. tuberculosis was added to each well. Cytokine measurement for murine interleukin (IL)-2 was performed by ELISA (R&D Systems).

Lactate measurements
Lactate was measured from cell culture supernatants using a coupled enzymatic assay in which lactate was oxidised and the resulting H_2O_2 was coupled to the conversion of Amplex® Red reagent to fluorescent resorufin by HRP (horseradish peroxidase). 30 µL of lactate standard or 200-fold diluted sample was added to a black 96-well flat-bottom plate followed by 30 µL of reaction mix which consisted of 0.6 µL of 10 U/mL HRP (Sigma), 0.6 µL of 100 U/mL lactate oxidase (Sigma), 0.3 µL of 10 mM Amplex® Red reagent (Life Technologies) and 28.5 µL PBS. The assay was incubated for 20 min at room temperature (RT) and the fluorescence of resorufin (excitation/emission maxima=570/585 nm) was measured on a 96-well plate reader (Biotek).

Transcriptional analysis of isolated PBMCs
RNA was isolated from unstimulated PBMCs using TRizol reagent (Invitrogen), according to the manufacturer’s protocol. RNA was transcribed into complementary DNA by reverse-transcription using iScript cDNA synthesis kit (BIORAD). Quantitative real-time PCR (qPCR) was performed using different primer sets (Biolegio); primer sequences for hexokinase 2 (HK2), HK3, 6-Phosphofructo-2-Kinase/ Fructose-2,6-Biphosphatase 3 (PFKFB3), Pyruvate Dehydrogenase Kinase 4 (PDK4), Malate Dehydrogenase 1 (MDH1), MDH2, Toll Like Receptor 2 (TLR2), TLR4, Nucleotide Binding Oligomerization Domain Containing 2 (NOD2), Caspase Recruitment Domain Family Member 9 (CARD9), Receptor Interacting Serine/Threonine Kinase 2 (RIPK2), Mitogen-Activated Protein Kinase 9 (MAPK9), TNF Receptor Associated Factor 6 (TRAF6), Caspase 1 (CASP-1) are given in Supplementary Table 1. Power SYBR Green
PCR Master Mix (Applied Biosystems) was used for qPCR in a AB Step one plus real-time PCR system (Applied Biosystems, Life technologies). qPCR data were normalized to the housekeeping gene human β2M.

**Statistics**

Data are shown as means ± SEM. Differences in cytokine secretion were calculated using the Mann-Whitney test for two independent samples. Correlation analysis between glucose metabolites and cytokine secretion was performed using Spearman’s rank correlation coefficient and the 95% confident interval was calculated accordingly. A p-value of <0.05 was considered statistical significant. Generation of graphs and statistical analyses were performed using Graphpad prism 5.
RESULTS

Participant characteristics are shown in Table 1. Age, plasma triglyceride (TG) and free fatty acid (FFA) levels were not different between T1D patients and the 24 control subjects (Table 1). As per definition, HbA1c and plasma glucose levels were significantly increased in T1D patients compared to healthy controls. Plasma insulin levels were also higher, whilst plasma cholesterol levels were lower in T1D patients.

| Table 1 | Descriptive characteristics of study type 1 patients (T1D) and controls (CON) |
|---------|-----------------|-----------------|---------|
|         | T1D patients    | Controls        | P-value |
| N       | 24              | 24              |         |
| Age (yr)| 48.1 ± 2.7      | 47.5 ± 2.6      | 0.91    |
| Duration diabetes (yr)| 24.2 ± 2.5 | - | - |
| Glucose (mmol/l)| 9.3 ± 0.8 | 4.6 ± 0.2 | <0.001*** |
| HbA1c (%)| 8.9 ± 0.3 | 5.4 ± 0.1 | <0.0001*** |
| Insulin (mE/l)| 3.5 ± 0.3 | 4.0 ± 0.1 | <0.05* |
| Cholesterol (mmol/l)| 1.36 ± 0.15 | 1.25 ± 0.12 | 0.70 |
| TG (mmol/l)| 0.25 ± 0.04 | 0.25 ± 0.04 | 0.39 |

Data are mean ± SEM. FFA, free fatty acids; HbA1c, glycosylated haemoglobin; TG, triglycerides.

PBMCs of T1DM patients show reduced pro-inflammatory IL-1β cytokine secretion in response to *M. tuberculosis*. No spontaneous cytokine production was detected in unstimulated TID patients or control cells (RPMI; Fig1). In contrast, robust induction of cytokine production was observed in response to *M. tuberculosis* in both groups. Production of IL-1β was significantly lower in T1D patients as compared to healthy controls (p < 0.01; Fig1). IL-6 and IFN-γ followed a similar trend, albeit borderline significance (p = 0.06). TNF-α, IL-1ra, IL-17 and IL-22 levels were not different between T1D patients and controls (Fig1). The decrease in IL-1β production was not specific to *M. tuberculosis* stimulation. Six additionally recruited T1D patients also produced lower levels of IL-1β in response to high dose LPS stimulation (Supplementary Fig1).
Figure 1 | PBMCs from T1D subjects secrete less IL-1β upon stimulation with M. tuberculosis.

Peripheral blood mononuclear cells (PBMCs, 5x10^5/well) from type 1 diabetes patients and healthy control subjects were stimulated with 1 µg/mL M. tuberculosis lysate. Secretion of (A) interleukin (IL)-1β, (B) IL-6, (C) IL-1Ra and (D) tumor necrosis factor (TNF)-α were measured in supernatants by ELISA after 24h h of stimulation. (E) Interferon (IFN)-γ, (F) IL-17 and (G) IL-22 were measured after 7 d of stimulation. Data are mean ± SEM from n=24 individuals per group. ** p <0.01 compared with matched healthy controls.

Correlation of IL-1β cytokine secretion with glycaemia. To determine whether the lower cytokine production in PBMCs from T1D patients in response to M. tuberculosis stimulation was related to glucose control cytokine production was correlated with HbA1c and glucose. However, there were no correlations between M. tuberculosis-induced IL-1β, IL-6 or IFN-γ secretion from PBMCs and HbA1c or plasma glucose levels of T1D patients (Fig2). Also, no correlations were found between cytokine secretion and duration of diabetes (data not shown). IL-1β secretion strongly correlated with IL-6 secretion in response to M. tuberculosis, but there were no other correlations between cytokine responses.
Figure 2 | Correlation of cytokine secretion with glycaemia. Associations for T1D patients between glycated hemoglobin (HbA1c%), plasma glucose levels and secretion of interleukin (IL)-1β, IL-6 and interferon (IFN)-y from PBMCs after stimulation with 1 µg/mL M. tuberculosis lysate. (A) Spearman’s rank correlation coefficients for all associations. (B) Correlations are shown by linear fitted curves and 95% confident intervals for T1D subjects (n=24).

Influence of serum and cellular metabolism on cytokine production. The cross-over of autologous serum from control to T1D and vice versa did not influence cytokine production of either subject group (Fig3). This suggests that the reduced IL-1β secretion is not directly related to plasma glucose concentration or other serum factors, instead it is likely to be an intrinsic defect within the immune cells of T1D patients.
Figure 3 | Influence of T1D serum on cytokine production in response to *M. tuberculosis*.

(A) Peripheral blood mononuclear cells (PBMCs, 5x10^5 cells/well) of healthy control (CON) subjects were incubated in either 25% of autologous serum or 25% of serum from an age-matched type 1 diabetes (T1D) subject and vice-versa for PBMCs from T1D subjects. Secretion of (A) interleukin (IL)-1β, (B) IL-6, (C) tumor necrosis factor (TNF)-α were measured in the supernatants using ELISA after 24 h of exposure to *M. tuberculosis*. (D) Interferon (IFN)-γ, (E) IL-22 and (F) IL-17 were determined after 7 d. Data are mean ± SEM from n=24 individuals per group. *P<0.05 compared with healthy controls.

Activation of aerobic glycolysis is important for cytokine production in response to *M. tuberculosis* 10. However, lactate production, a marker of glycolysis, was not decreased in T1D patients. In fact, *M. tuberculosis* stimulation increased lactate production in T1D patients compared to matched healthy controls (Fig4A). Glucose consumption (Fig4B) and the expression levels of glycolysis genes HK2, HK3 and PFKFB3 in PBMCs was similar between T1D patients and healthy controls (Fig4 E –G). Finally, no difference in the expression of the TCA cycle genes: PDK4, MDH1 and MDH2 (Fig4 H-J) was observed.
Figure 4 | Cellular metabolism in PBMCs from T1D subjects and controls. (A) Lactate secretion and (C) glucose consumption were measured in the supernatant of unstimulated (RPMI) peripheral blood mononuclear cells (PBMCs) of type 1 diabetes (T1D) and healthy control (CON) subjects as well as (B, D) after 24 h of stimulation with *M. tuberculosis* lysate. Relative mRNA expression values of (E) HK2, (F) HK3, (G) PFKFB3, (H) PDK4, (I) MDH1 and (J) MDH2 in unstimulated PBMCs from T1D and CON subjects. Data are mean ± SEM from n=24 individuals per group.
Figure 5 | *M. tuberculosis* recognition and downstream signalling. Relative basal mRNA expression values of peripheral blood mononuclear cells (PBMCs) from type 1 diabetes (T1D) are shown when compared to matched healthy control (CON) for (A) TLR2, (B) TLR4, (C) NOD2, (D) CARD9, (E) RIPK2, (F) MAPK9, (G) TRAF6 and (H) CASP1. Data are mean ± SEM from n=24 individuals per group.
**M. tuberculosis recognition and downstream signalling.** To determine whether changes in receptors involved in *M. tuberculosis* recognition could explain the impaired cytokine response of PBMCs from T1D patients, we investigated gene expression levels of well-known *M. tuberculosis* pattern recognition receptors (PRRs) in PBMCs of both groups. Levels of TLR2, TLR4 and NOD2 were unchanged (Fig5 A-C). In addition, no differences were found in the expression of genes involved in the intracellular signalling response to *M. tuberculosis*, including CARD9, RIPK2, MAPK9, TRAF6 and CASP1 (Fig5D-H).

**Bioactive IL-1 secretion from PBMCs of T1D patients.** To determine whether differences in cellular processing affected the secretion of IL-1β, we determined the amount of bioactive IL-1 in the supernatants of PBMCs stimulated with *M. tuberculosis* from Fig1. We found that PBMCs of T1D patients released significantly lower levels of bioactive IL-1 in response to *M. tuberculosis*.

**Figure 6 | Bioactive IL-1 secretion from PBMCs of T1D patients.** Peripheral blood mononuclear cells (PBMCs, 5x10^5/well) from type 1 diabetes patients and healthy control subjects were stimulated with 1 µg/mL *M. tuberculosis* lysate for 24 h and supernatants were subsequently incubated with the murine thymoma EL4-NOB1 cell line (10^5 cells/well). Mouse interleukin (IL)-2 cytokine secretion in response to present bioactive IL-1 in the supernatant of the PBMCs was measured using ELISA. Data are mean ± SEM from n=24 individuals per group. *P<0.05 compared with the matched healthy controls.
DISCUSSION

The primary finding of this study is that PBMCs from patients with T1D have a reduced ability to produce IL-1β, IL-6 and IFN-γ in response to *M. tuberculosis* stimulation, while TNF, IL-17 and IL-1Ra production is normal. These changes may be partly responsible for the well-known susceptibility of patients with diabetes for TB. The current data suggest that the decreased cytokine production after stimulation with *M. tuberculosis* is an intrinsic cellular deficit in PBMCs from T1D patients, since differences in cytokine secretion were not due to external factors in serum or related to glucose regulation.

Epidemiological studies that investigated the relation between hyperglycaemia and the risk for TB have been contradicting. Both a positive effect\(^5,6\) and no effect\(^11,12,13\) of hyperglycaemia on the risk for TB have been described. These studies vary greatly in size, geography and definition of controlled vs uncontrolled glycaemia. To our knowledge this is the first study specifically examining the cytokine production capacity of T1D patients to *M. tuberculosis*. At the same time, studies on the cytokine response to other pathogenic stimuli such as LPS strongly support our findings. Ex vivo stimulations experiments performed in both T1D\(^14,15,16\) and T2D\(^17,18\) patients describe a similar decrease of IL-1β and IL-6 but not TNF-α, IL-1ra and IFN-γ production, respectively. As shown in the stimulation experiments with LPS, this decrease in cytokine signalling is not specific for *M. tuberculosis*, thus supporting the notion that an intrinsic defect in cytokine production rather than an *M. tuberculosis* specific signalling defect is the mechanism underlying the decrease in cytokine production.

Interestingly, patients with both tuberculosis and diabetes produce more cytokines compared to patients with only tuberculosis\(^19,20\). Together, these findings suggest an initial sub-optimal response to infection which promotes bacterial growth and subsequent pathology. This hypothesis of a delayed immune response is supported by a mouse model of concomitant tuberculosis and diabetes disease\(^21\).

The exact type of intracellular defects remains to be elucidated. No change in aerobic glycolysis was observed nor altered expression of relevant pattern recognition receptors. It is possible that a certain level of accelerated immunosenescence occurs in T1D. This process, described for ageing, is characterized by impaired cellular immune function (leading to increased death due to infectious diseases), simultaneously with increased chronic inflammatory activity (leading to increased incidence of cardiovascular disease)\(^22\). The observations that T1D patients show enhanced low-grade chronic inflammation and cardiovascular diseases\(^23\) and at the same time an impaired immune response to pathogens as observed in the current study support this hypothesis. The decrease in IL-6 may be a secondary effect as IL-1β induces IL-6 production in a paracrine signaling loop. Similarly,
IL-1β, another product of caspase-1 activity, is important for IFN-γ production. This could explain why the decrease in these cytokines is more subtle as compared to IL-1β. This avenue will form a cornerstone for further research.

The implications of a decrease in IL-1β production for susceptibility to TB are significant. IL-1α/β double knockout mice or mice deficient in IL-1R1 display increased susceptibility to *M. tuberculosis* infection. In humans, a positive association between IL-1β levels and resistance to tuberculosis was inferred from human *IL1B* gene polymorphisms. Moreover, patients with gain of function mutations in the inflammasome component NLRP3 have an increased capacity to kill *M. tuberculosis*. Mechanistically, a recent study elucidated that IL-1β promoted bacterial containment through the induction of specific eicosanoids that limited excessive and type 1 IFN production. The current study was performed in T1D patients, with chronic hyperglycemia as a key characteristic. The observed impaired cellular cytokine response could be similar for T2D patients. However, T2D is a much more heterogeneous disease characterized by multiple metabolic disturbances (i.e. hyperglycaemia, insulin resistance, hypertension, dyslipidemia and oxidative stress) which may further contribute to the increased susceptibility to TB and which needs further investigation.

As a limitation of this study, we did not investigate other host defense mechanisms such as phagocytosis, ROS or nitric oxide (NO) production from monocytes or macrophages since it is unlikely that reduced IL-1β is the sole factor in susceptibility to TB. A confounding factor in the current study could be the significantly higher levels of insulin in blood of T1D patients as insulin can affect cytokine production however, we did not find any correlation between insulin and cytokine production in T1D patients (data not shown). Lastly, a limitation of this study is the relatively small size of our cohort as a result of which subtle associations such as those between glucose control and cytokine responses may not be detectable.

In conclusion, this study specifically identifies a decrease in bio-active IL-1β possibly, due to a deficit in intracellular processing rather than chronic hyperglycaemia, to be a strong candidate in the susceptibility of T1D patients to tuberculosis.
REFERENCES


SUPPLEMENTARY MATERIAL

Supplementary Figure 1 | Reduced IL-1β secretion by PBMCs from T1D subjects is not specific to M. tuberculosis stimulation. Blood peripheral mononuclear cells (PBMCs) of six additional type 1 diabetes (T1D) subjects and healthy control (CON) subjects have been challenged with low and high dose of lipopolysaccharide (LPS, 1ng/mL and 10ng/mL) for 24 h and interleukin (IL)-1β secretion was measured using ELISA. Data are mean ± SEM from n=6 individuals per group.

Supplementary Table 1 | Primer sequences used for qRT-PCR analysis (5’-3’)

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<td>CASP-1</td>
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Chapter 4

Diabetes mellitus and increased tuberculosis susceptibility: the role of short-chain fatty acids

Ekta Lachmandas
Corina N.A.M. van den Heuvel
Michelle Damen
Maartje C.P. Cleophas
Mihai G. Netea
Reinout van Crevel
ABSTRACT

Type 2 diabetes mellitus confers a three-fold increased risk for tuberculosis, but the underlying immunological mechanisms are still largely unknown. Possible mediators of this increased susceptibility are short-chain fatty acids, levels of which have been shown to be altered in individuals with diabetes. We examined the influence of physiological concentrations of butyrate on cytokine responses to *Mycobacterium tuberculosis* (Mtb) in human peripheral blood mononuclear cells (PBMCs). Butyrate decreased Mtb-induced pro-inflammatory cytokine responses, while it increased production of IL-10. This anti-inflammatory effect was independent of butyrate’s well-characterised inhibition of HDAC activity, and was not accompanied by changes in Toll-like receptor signalling pathways, the eicosanoid pathway or cellular metabolism. In contrast blocking IL-10 activity reversed the effects of butyrate on Mtb induced inflammation. Alteration of the gut microbiota, thereby increasing butyrate concentrations, can reduce insulin resistance and obesity, but further studies are needed to determine how this affects susceptibility to tuberculosis.
INTRODUCTION

Tuberculosis (TB) is the second leading cause of death from an infectious disease worldwide [1]. Susceptibility to TB can be increased by several co-morbidities, one of which is type 2 diabetes mellitus (DM) [2]. DM patients present with an overall three-fold increased risk of developing active TB [3]. Globally, 15% of TB cases are estimated to be attributable to DM [4], and with a predicted increase of DM by 155% over the next 20 years, DM will become an increasingly important factor challenging TB control [5-7].

DM patients exhibit alterations in the immune response against Mycobacterium tuberculosis (Mtb), making them more susceptible to infection or progression towards active TB disease, and less responsive to treatment [8-11]. However, the underlying biological mechanisms remain largely unknown [12, 13]. DM patients have been associated with dysregulated cytokine responses to Mtb [14-17]. While pro-inflammatory cytokines are necessary for protection against Mtb, anti-inflammatory cytokines may counteract these effects. Possible factors that may impact the host response in patients with DM are short-chain fatty acids (SCFAs), the main metabolic products of fermentation of non-digestible dietary fibres by the gut microbiota. Numerous reports have demonstrated that DM patients present with an altered composition of their gut microbiota, which subsequently alters their SCFA levels [18-24]. SCFAs strongly modulate immune and inflammatory responses [22, 25-31], thereby influencing the host response to Mtb. SCFAs, of which butyrate (C4) is the most thoroughly studied, act on immune and endothelial cells via at least two mechanisms: activation of G-protein coupled receptors (GPCRs) and inhibition of histone deacetylase (HDAC) [32]. They affect the function of various cell types such as lymphocytes [33, 34], neutrophils [25, 31, 35], and macrophages [28, 36-38]. In light of the emerging role of the microbiota in inflammation and immunity we hypothesized that SCFAs, and in particular butyrate, may affect the immune response and susceptibility to Mtb in type 2 DM patients.

In this study we investigated the role of physiological concentrations of SCFAs on the cytokine response against Mtb in human peripheral blood mononuclear cells (PBMCs). We subsequently examined a number of possible mechanisms via which altered concentrations of one particular SCFA, C4, might affect the host immune response to Mtb in DM patients. To this purpose, we studied the influence of physiological concentrations of C4 on HDAC activity, immune signalling pathways, the eicosanoid pathway and cellular metabolism. To our knowledge, this is the first study reporting on the effects of physiological plasma concentrations of C4 on Mtb-induced cellular responses. Physiological plasma concentrations of C4 are in the micromolar range [39], while in previous studies C4 was used in the millimolar range. Therefore, this study substantially adds to our knowledge of SCFAs as possible mediators of altered immune responses to Mtb in DM patients. The authors declare that there is no conflict of interest regarding the publication of this paper.
METHODS

Human samples
PBMCs were isolated from buffy coats donated after written informed consent by healthy volunteers to the Sanquin Bloodbank (http://www.sanquin.nl/en/) in Nijmegen. Experiments were conducted according to the principles expressed in the Declaration of Helsinki. Since blood donations were anonymous no tuberculosis skin test or IFN-γ release assay was performed. However the incidence of TB in the Dutch population is extremely low (4/100,000), and Bacillus Calmette-Guérin (BCG) vaccination is not part of the routine vaccination program. Blood donors were not screened for DM as prevalence of DM among people under 45 years of age (median age of blood donors) is about 1.5% and therefore DM is unlikely to be a confounding factor [34].

H37Rv lysates and culture
H37Rv Mtb was grown to mid-log phase in Middlebrook 7H9 liquid medium (Difco, Becton-Dickinson) supplemented with oleic acid/albumin/dextrose/catalase (OADC) (BBL, Becton-Dickinson), washed three times in sterile saline, heat killed and then disrupted using a bead beater, after which the concentration was measured using a bicinchoninic acid (BCA) assay (Pierce, Thermo Scientific).

Cell stimulation experiments
Isolation of PBMCs was performed by differential centrifugation over Ficoll-Paque (GE Healthcare). Cells were adjusted to \(5 \times 10^6\) cells/ml (Beckman Coulter) and suspended in RPMI 1640 (Gibco) supplemented with 10 µg/ml gentamicin (Lonza), 10 mM L-glutamine (Life Technologies), and 10 mM pyruvate (Life Technologies). 100 µl of PBMCs were incubated in round-bottom 96-well plates (Greiner), pre-treated with SCFAs for 1 h and stimulated with 1 µg/ml of H37Rv lysate or 10 ng/ml LPS (Sigma-Aldrich, E. coli serotype 055:B5. Cells were incubated for 24 h or 7 days at 37°C in a 5% CO\(_2\) environment (n = 6 to 11). Alternatively, PBMCs were pre-treated for 1 hour (37°C, 5% CO\(_2\)) with ranolazine (ITK-Diagnostics), trimetazidine (Sigma), pertussis toxin (Enzo Life Sciences), etomoxir (Sigma) (inhibitors of β-oxidation, n = 3), aspirin (Aspéigic injection powder, n = 3), cycloheximide (Sigma, n = 6 to 7), anti-IL-10 antibody IgG2a (BioLegend, n = 10 to 12), or IgG2a isotype control (Biolegend, n = 10 to 12) prior to stimulation. Cell culture supernatants were collected and stored at -20°C for cytokine measurements, performed by ELISA: TNF-α, IL-1β, IL-17A, IL-22, IL-1Ra (R&D Systems); IL-6, IFN-γ and IL-10 (Sanquin).
Quantification of gene expression

For quantitative real-time PCR (qPCR) analysis RNA was isolated from PBMCs using TRizol reagent (Invitrogen Life Technologies) according to the manufacturer’s protocol. RNA was transcribed into complementary DNA (cDNA) by reverse-transcription using iScript cDNA synthesis kit (BIORAD, Hercules, CA). Primer sequences (Biolegio) are given in Table 1. Power SYBR Green PCR Master Mix (Applied Biosystems) was used for qPCR on an AB Step one plus real-time PCR system (Applied Biosystems). qPCR data was normalized to the housekeeping gene human β2M (n = 3 to 10).

Table 1 | Primer sequences used for gene expression measurements by qPCR.

<table>
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<td>h-Tollip</td>
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Protein phosphorylation measurements

Western blotting was carried out using a Trans Turbo Blot system (Bio-Rad) according to manufacturer’s instructions. 5x10⁶ PBMCs were lysed in 100 μl lysis buffer. The resulting lysate was used for Western blot analysis. Equal amounts of protein were separated by SDS-PAGE on 4-15% polyacrylamide gels (Bio-Rad) and transferred to PVDF (Bio-Rad) membranes. Membranes were blocked for 1 hr and then incubated overnight with primary antibody
(dilution 1:1000) in 5 % (w/v) BSA or milk in TBS-Tween buffer (TBS-T). Blots were washed in TBS-T 3 times and incubated with HRP-conjugated anti-rabbit antibody (1:5000; Sigma) in 5% (w/v) milk in TBS-T for 1 h at room temperature (RT). After washing, blots were developed with ECL (Bio-Rad) following manufacturer’s instructions. Primary antibodies used were rabbit anti-p38 MAPK, rabbit anti-phospho p38 MAPK, rabbit anti-ERK1/2 (p44/p42 MAPK), rabbit anti-phospho ERK1/2 (P44/42 MAPK, T202/Y204) and rabbit anti-phospho-JNK (T183/Y185) (all Cell Signalling) (n = 2).

Metabolite measurements

Lactate was measured from cell culture supernatants using a coupled enzymatic assay in which lactate was oxidised and the resulting H₂O₂ was coupled to the conversion of Amplex® Red reagent to fluorescent resorufin by HRP (horseradish peroxidise). 30 μl of lactate standard or 200x diluted sample was added to 30 μl of reaction mix. The 30 μl of reaction mix consisted of 0.6 μl of 10 U/ml HRP (Sigma), 0.6 μl of 100 U/ml lactate oxidase (Sigma), 0.3 μl of 10 mM Amplex® Red reagent (Life Technologies) and 28.5 μl PBS. Samples were incubated for 20 min at RT and fluorescence (excitation/emission maxima=570/585 nm) was measured on an ELISA reader (Biotek) (n = 3 to 5).

Measurements of the NAD⁺/NADH redox ratio were adapted from Zhu et al [40]. Briefly, 1.5 million stimulated PBMCs were lysed in 75 μl of homogenization buffer (10 mM nicotinamide (Sigma), 10 mM Tris-Cl (Sigma), 0.05% (w/v) Triton X-100 (Sigma), pH 7.4). The lysate was centrifuged at 12000 g for 1 min. From the resulting supernatants two 18 μl aliquots were removed and either 2 μl of 0.2M HCl or 0.2 M NaOH was added to each aliquot. The samples were heated for 30 min at 65°C and after incubation 2 μl of opposite reagent (NaOH or HCl) was added to each aliquot. 5 μl of sample or NAD⁺ (β-nicotinamide adenine dinucleotide hydrate; Sigma) standard was then mixed with 85 μl of reaction mix and 60 μl of fluorescence mix in a black 96-well plate. The reaction mix consisted of 100 mM bicine (N,N-Bis(2-hydroxyethyl)glycine; Sigma), 0.6 mM ethanol (Sigma) and 5 mM EDTA (Life Technologies). The fluorescence mix consisted of 0.5 mM PMS (phenazine methosulfate; Sigma), 0.05 mM resazurin (Sigma) and 0.2 mg of ADH (alcohol dehydrogenase; Sigma). The reaction was incubated for 15 min at RT and fluorescence (excitation/emission maxima=540/586 nm) was measured on an ELISA reader (Biotek) (n = 3 to 5).

HDAC activity assay

HDAC Fluorimetric Cellular Activity Assay BML-AK503 (Fluor de Lys, Enzo Life Sciences, Inc., Farmingdale, VS) was used to determine HDAC activity in PBMCs pre-treated with C4 (30 min) and then stimulated with H37Rv (30 min). Subsequently PBMCs were incubated with
acetylated substrate for 2 hours, after which a developer was added to generate a fluorescent signal from the deacetylated substrate. Fluorescence was measured on a microplate reader (Biotek). Trichostatin A (TSA) was used as a positive control for HDAC inhibition (n = 5 to 6).

**Flow cytometry**

PBMCs were treated with 50 µmol C4 for 1 h and stimulated with 1 µg/ml H37Rv or 10 ng/ml LPS for 7 days. Subsequently cells were re-stimulated with 200 µL RPMI supplemented with 10% serum, Golgi-plug inhibitor (GPI Brefeldin A; 1 µg/ml, BD Pharmingen), PMA (phorbol 12-myristate 13-acetate; 50 µg/ml, SIGMA Aldrich) and ionomycin (1 µg/ml, SIGMA Aldrich) for 4-6 h at 37°C and 5% CO₂. Cells were then washed with PBA (PBS 1% BSA (albumin from bovine serum) and stained extra-cellularly for 30 min with CD4-PeCys7 (ITK) for T-helper 17 (Th17) cells at 4°C. Next, cells were washed and permeabilized by fix and perm buffer (e-bioscience) according to the manufacturer’s protocol for 45-60 min at 4°C. Finally cells were washed and re-suspended in 300 µL PBA to be measured using the Cytomics FC500 (Beckman Coulter) (n = 8).

Cell death was measured by staining PBMCs with Annexin V-FITC (BioVision) and Propidium Iodide (PI) (Invitrogen Molecular Probes). Cells were incubated in the dark on ice with Annexin-V staining solution (RPMI supplemented with 5 mM CaCl₂ and 0.1 µl/ml Annexin-V) for 15 minutes. Subsequently PBMCs were stained with PI for 5 minutes. Cells were measured with the Cytomics FC500 (Beckman Coulter, Woerden, The Netherlands), and data were analysed using CXP analysis software v2.2 (Beckman Coulter) (n = 3 to 5).

**Statistical analysis**

All data were analysed using a paired non-parametric Wilcoxon signed rank test, as the data were not-normally distributed. Differences were considered statistically significant at a p-value <0.05. Data are shown as cumulative results of levels obtained in all volunteers (means ± SEM).
RESULTS

Short-chain fatty acids inhibit Mtb-induced cytokine responses. DM is associated with altered gut microbiota and consequently altered SCFA levels [18-22]. In line with current literature [22, 25-31], we hypothesized that SCFAs have the potential to influence the host inflammatory response against Mtb. In particular we investigated the effects of varying doses of acetate (C2), propionate (C3), and butyrate (C4) on H37Rv-induced cytokine responses, with RPMI as negative control and LPS as positive control (Figure 1). SCFAs themselves did not induce cytokine production (results not shown), but significantly affected H37Rv-induced cytokine release. C2, C3 and C4 significantly, dose-dependently decreased H37Rv-induced production of pro-inflammatory cytokines TNF-α, IL-1β, and IL-17, while non-significant effects were found for IL-6, IFN-γ, and IL-22 production. In contrast, C3 and C4 induced a significant increase in H37Rv-induced production of the anti-inflammatory cytokine IL-10. Similarly, C3 and C4, but not C2 decreased LPS-induced production of TNF-α and IL-6, while the release of IL-1β was significantly decreased in response to all three SCFAs (results not shown). LPS did not induce production of IFN-γ, IL-17, or IL-22. Moreover, all three SCFAs incurred a dose dependent, non-significant decrease in LPS-induced IL-10 production (results not shown).

Overall, C4 resulted in some of the most significant changes in cytokine responses (Figure 1 B). Moreover, the potency of butyrate in reducing cytokine responses to H37Rv and LPS was greater than for the other SCFAs. Importantly, changes in cytokine levels could not be explained by altered pH levels or cell death (Supplementary Figure 1 A and B). Therefore, following this screen we continued our study with C4 at a concentration of 50 µM, which is physiologically relevant because it is comparable to human plasma concentrations [39].

Influence of butyrate on HDAC expression and activity. Butyrate is reported to be a strong HDAC inhibitor. Because this might account for its anti-inflammatory effects [41-44], we examined the effect of C4 on HDAC expression and activity. C4 significantly decreased HDAC8 but not HDAC1 gene expression upon H37Rv stimulation of PBMCs (Figure 2 A). Consistent with previous reports [36, 42-44], C4 at a high dose of 1 mM decreased HDAC activity upon both RPMI and H37Rv stimulation. However, different from its effect on gene expression, C4 at a physiological dose of 50 µM had no effect on actual HDAC activity (Figure 2 B), while trichostatin A (TSA, positive control) strongly decreased HDAC activity. These data suggest that butyrate’s inhibition of HDAC activity is unlikely to play a role in the effects of low doses of C4 on Mtb-induced inflammatory responses and stresses the importance of studying the effects of butyrate at physiologically relevant concentrations.
Figure 1 | Short-chain fatty acids inhibit Mtb-induced cytokine responses. (A) PBMCs were pre-incubated with 2-250 μM SCFAs for 1 h prior to stimulation with Mtb lysate for 24 h and 7 d. Hereafter TNF-α, IL-6, IL-10, IFN-γ, IL-17 and IL-22 were measured in supernatants by ELISA. Data are means ± SEM (n = 6), Wilcoxon signed rank test, representative of 2 independent experiments. * p < 0.05 (B) Heatmap of log-transformed mean cytokine responses as measured by ELISA, showing cytokines up-regulated (red) and down-regulated (blue) upon H37Rv stimulation in the presence of different doses of SCFAs. Cytokine responses are shown as compared to H37Rv stimulation alone. * p < 0.05
The effects of butyrate on TLR-signalling mediators and the eicosanoid pathway.

Signalling of Toll-like receptors (TLRs), important receptors for Mtb recognition [45-47], is controlled by feedback mechanisms regulated by several intracellular kinases [48, 49]. Because impaired Mtb recognition and insufficient TLR signalling may account for the anti-inflammatory effects of C4, we examined whether C4 affects these feedback loops. However, C4 had no effect on phosphorylation of the MAP kinases p38, ERK (Figure 3 A), or JNK (Supplementary Figure 2). C4 has also been reported to induce expression of inhibitors of
TLR-signalling pathways [50], but we found that C4 significantly decreased mRNA expression of TLR-signalling inhibitors SOCS1 and Tollip, and did not affect expression of SOCS3 or ST2 (Figure 3 B). Of note, these results were not explained by cell death (Supplementary Figure 1 B).

Aside from TLR-signalling, C4 possibly exerts its anti-inflammatory effects through modulation of the eicosanoid pathway. Eicosanoids, oxygenated metabolites of arachidonic acid, modulate the host immune response to Mtb [51-55]. C4 has been reported to up-regulate key enzymes of the eicosanoid pathway upon LPS stimulation [30], but a reversed effect has also been described [56]. We did not observe a significant impact of C4 on transcript levels of cyclo-oxygenase 2 (COX-2), one of the main eicosanoid enzymes, upon H37Rv or LPS stimulation (Supplementary Figure 3 A). Alternatively, C4 has been described to induce release of the anti-inflammatory prostaglandin PGE$_2$ [26, 30, 57]. Inhibition of PGE$_2$ with aspirin could not counteract the inhibitory effects of C4 on TNF-α and IL-1β cytokine responses upon either H37Rv or LPS stimulation (Supplementary Figure 3 B). The eicosanoid pathway is therefore unlikely to be the mediator pathway through which C4 exerts its anti-inflammatory effects.

Influence of butyrate on cellular metabolism. Another possible explanation for butyrate’s anti-inflammatory effects is its influence on cellular metabolism. A recent paper described that microbiota have a strong effect on energy homeostasis in the mammalian colon, and showed that C4 regulates different aspects of energy metabolism acting as an important energy source for colonocytes [58]. Contrary to this previous study, we observed no effects of C4 on cellular lactate production, the NAD$^+/NADH$ redox ratio, TCA cycle gene expression (Figure 4); nor on β-oxidation (Supplementary Figure 4). These data strongly suggest that C4 modulates the immune response to Mtb independently of cellular metabolism.
Figure 3 | The effects of butyrate on TLR-signalling mediators. (A) PBMCs were pre-incubated with 50 µM C4 (1 h) and stimulated with Mtb lysate or LPS. Cell lysates were harvested at 30 min post stimulation. Phospho-p38, p38, phospho-ERK, and ERK protein levels were determined by western blot using specific antibodies (n = 2). (B) Gene expression levels of SOCS1, SOCS3, ST2, and Tollip in PBMCs pre-incubated with 50 µM C4 (1 h) and stimulated with Mtb lysate or LPS (4 h) as measured by qPCR. The boxplot represents median with first and third quartiles; the whiskers represent minimum and maximum values. n = 10, Wilcoxon signed rank test, representative of 3 independent experiments. * p < 0.05
Figure 4 | Influence of butyrate on cellular metabolism. (A and B) Kinetics of lactate production (A) and intracellular NAD+/NADH ratios (B) from days 1, 3 and 7 of PBMCs pre-incubated with 50 µM C4 (1 h), with and without stimulation with Mtb lysate. Data are means ± SEM (n = 3 to 5), Wilcoxon signed rank test, representative of 1-2 independent experiments. (C) Expression levels of glycolysis and TCA cycle genes in PBMCs pre-incubated with 50 µM C4 (1 h) and stimulated with Mtb lysate or LPS (4 h) as measured by qPCR. Data are means ± SEM (n = 6), Wilcoxon signed rank test, representative of 2 independent experiments.
Butyrate transcriptionally influences cytokine responses to Mtb, possibly mediated through IL-10 induction. We next examined whether the inhibitory effect of C4 on Mtb-induced pro-inflammatory cytokine responses, with a concomitant increase in anti-inflammatory IL-10 production (Figure 1) and decrease in Th17 proliferation (Supplementary Figure 5 A), was also present at the level of gene transcription. C4 led to a decrease in TNF-α, IL-12 and IL-23 mRNA levels upon H37Rv stimulation, and a parallel increase in IL-10 mRNA (Figure 5 A), while no effect on production of the anti-inflammatory cytokine IL-1Ra was observed (Supplementary Figure 5 B). These data point to IL-10 as a possible intermediary mediator of the anti-inflammatory effects of C4. We therefore assessed whether removing IL-10 protein from the cellular environment could counteract the inhibitory effects of C4. To this end, we pre-treated PBMCs with cycloheximide (CHX), an inhibitor of translation. Stimulation of PBMCs with H37Rv in the presence of C4 in combination with CHX resulted in higher TNF-α responses, as compared to incubation with H37Rv and C4 alone. Upon LPS stimulation, this effect was not present (Figure 5 B). We subsequently examined whether blocking IL-10 specifically using an anti-IL-10 antibody could counteract the inhibitory effects of C4 on pro-inflammatory cytokine response. Blocking IL-10 completely restored IL-6 cytokine responses in response to H37Rv and C4, while TNF-α and IL-1β production was partly restored (Figure 5 C). This suggests an important role for intermediary protein synthesis, specifically IL-10, in mediating the anti-inflammatory effects of C4.
Figure 5 | Butyrate transcriptionally influences cytokine responses to Mtb, possibly mediated through IL-10. (A) Cytokine gene expression levels in PBMCs pre-incubated with 50 µM C4 for 1 h prior to stimulation with Mtb lysate or LPS for 4 h, as measured by qPCR. The boxplot represents median with first and third quartiles; the whiskers represent minimum and maximum values. n = 6 to 10, Wilcoxon signed rank test, representative of 2+ independent experiments. * p < 0.05. (B) To block translation PBMCs were pre-incubated with cycloheximide (CHX) for 1 h, prior to 1 h incubation with C4 (50 µM). TNF-α transcript levels were measured by qPCR 4 h after stimulation with Mtb lysate or LPS. Data are single values (n = 6 to 7), Wilcoxon signed rank test, representative of 3 independent experiments. * p < 0.05. (C) To block IL-10 activity, PBMCs were pre-incubated with IL-10 and C4 (50 µM) for 1 h. IL-6, TNF-α, and IL-1β production was measured by ELISA after 24 h of stimulation with Mtb lysate. Data are means ± SEM (n = 10 to 12), Wilcoxon signed rank test, representative of 4 independent experiments.
DISCUSSION

Diabetes is associated with a three-fold increased risk of active TB, but the underlying immunological mechanisms remain largely unknown [3, 12, 13]. Alterations in the gut microbiota of DM patients are associated with changes in plasma SCFA concentrations. Multiple papers have reported a decrease in C4-producing bacteria in type 2 DM patients [18, 19, 21, 23, 24]. We here show that SCFAs, especially C4, exhibit anti-inflammatory properties; low doses of C4 decreased Mtb-induced pro-inflammatory cytokine responses both on the transcriptional and translational level, while production of IL-10 was increased. This anti-inflammatory effect was independent of HDAC activity, Toll-like receptor signalling, the eicosanoid pathway, or cellular metabolism.

We observed a general anti-inflammatory effect of C2, C3, and C4 on Mtb-induced cytokine production. C4 induced some of the most significant and most potent changes in cytokine responses, which is in line with published results [29], although our study is the first to examine the effects of physiological concentrations of SCFAs on Mtb-induced cytokine responses in vitro. Several observations were made regarding the effect of SCFA on cytokines. Firstly, the inhibitory effect of all three SCFAs on production of TNF-α and IL-1β was comparable for Mtb and LPS stimulation. However, while C3 and C4 had a clear effect on LPS-induced IL-6 release, this was not found for Mtb. This suggests that SCFAs do not affect Mtb-induced IL-6, although IL-6 has been assigned an important role in Mtb host responses [59-62]. Secondly, C2, C3, and C4 had a much stronger inhibitory effect on T-cell derived cytokine IL-17, than on T-cell derived cytokines IFN-γ and IL-22. Because C4 also strongly decreased Th17 proliferation (Supplementary Figure 5 A), SCFAs may affect Th17 subsets more than other T-cell subsets. This may be of great relevance since Th17 cells, and IL-17 in particular, have been reported to be essential in protective immunity against Mtb [63, 64], but inversely associated with DM complications [65-67]. Lastly, the stimulatory effect of C3 and C4 on anti-inflammatory IL-10 release was Mtb-specific and not seen with LPS-stimulation. IL-10 has been delineated as an important mediator in Mtb infection: it has been reported to block bacterial killing in Mtb-infected macrophages, suppress multinucleated giant cell formation and cytokine production, and inhibit the development of protective immunity [68-74]. In contrast to TB, IL-10 may have a protective role in type 2 DM, by reducing insulin resistance and obesity [75-77]. Therefore, the increase in IL-10 production we see as induced by C4 is very relevant for the course of both DM and TB disease.

We examined several possible mechanisms underlying the effect of C4 on cytokine production, starting with HDAC activity, which is known to be inhibited by SCFAs. C4 at a physiological low dose of 50 µM had little effect, while millimolar concentrations of C4
SCFAs in TB-DM

(as used in other studies [36, 41-44]) decreased HDAC activity upon H37Rv stimulation. This is expected as the IC50 values of HDAC inhibition by C4 are > 100 µM, depending on the class of HDAC [43]. The strongest effect was noted for HDAC8, which is reported to be most sensitive to C4 [43]. This argues that physiological C4 concentrations in human plasma do not exert HDAC inhibition, and underlines the importance of using physiological concentrations within in vitro experimental models.

In contrast to a previous study [50], we observed a decreased gene expression of the TLR-modulatory factors SOCS1 and Tollip when PBMCs were stimulated in the presence of C4, which thus cannot explain the inhibitory effects on cytokine production. This, together with our data showing that C4 does not affect MAP kinase activity, suggests that C4 does not act at the level of TLR signalling, as shown previously [36].

As a third possible mechanism, we assessed whether C4 exerts its effects through eicosanoid metabolism. The eicosanoid pathway is under influence of SCFAs [30, 56] and may modulate the host response to Mtb [51-55]. C4 did not affect expression of COX-2, a key enzyme in the eicosanoid pathway, in contrast to previous reports that used supraphysiological C4 concentrations [30, 56]. In addition, inhibition of the eicosanoid pathway using aspirin did not counteract the effects of C4. Therefore, the eicosanoid pathway is unlikely to be involved in mediating the effects of C4.

The effect of diabetes on the host immune response to Mtb might also be explained by altered cellular metabolism, with a possible role for SCFA. Cellular metabolism is increasingly linked to immunology [78-80]. One previous study noted that C4 influences metabolic processes in colonocytes [58], which use butyrate as their primary energy source [58]. However, we did not observe any effect of C4 on lactate production, the redox status, TCA cycle gene expression, or β-oxidation in PBMCs. We therefore conclude that cellular metabolism does not mediate the effect of C4 on Mtb-induced cytokine production.

Finally, we further examined the effect of C4 on the anti-inflammatory cytokine IL-10. IL-10 is detrimental to TB outcome, while it may improve DM symptoms [68-77]. In line with previous studies [33, 81, 82], we report an up-regulation in IL-10 production induced by C4. Removal of all intermediary protein, including IL-10, from PBMCs stimulated with H37Rv and C4 led to a significant increase in TNF-α transcript, thereby counteracting the decrease in TNF-α production induced by C4. Moreover, blocking IL-10 specifically fully restored IL-6 responses in PBMCs stimulated with H37Rv and C4, and partly restored TNF-α and IL-1β responses. These data suggest that the anti-inflammatory cytokine IL-10 may play a role in the inhibitory effects of C4 on Mtb-induced inflammatory responses.
Currently, much research is focused on modulation of the gut microbiota in order to treat obesity and type 2 DM [83-86]. Administration of sodium butyrate or butyrate-inducing probiotics in mice significantly increased plasma insulin levels and insulin sensitivity and suppressed body weight gain [87-89]. The anti-inflammatory effects of C4 may attenuate the chronic inflammatory state associated with type 2 DM, thereby improving DM symptoms. If chronic inflammation is a causal factor of the impaired host response to Mtb in type 2 DM patients, attenuation of this hyper-inflammatory state may not only improve DM but also TB outcome in patients with coincident DM and TB disease.

Some limitations of our study need to be addressed. Firstly, we studied the effects of C4 on Mtb-induced inflammation in PBMCs \textit{in vitro}. SCFA levels have been shown to be altered in DM patients [18-22], but this \textit{in vitro} model does not include other aspects of the pathophysiology of DM such as hyperglycemia, hyperinsulinemia, or dyslipidemia, phenomena which have also been reported to affect immunity [90-94]. Furthermore, DM medications possibly interfere with the intestinal microbiota and immune responses in patients [95-97]. It is therefore unclear how accurately our \textit{in vitro} model reflects the \textit{in vivo} situation in DM patients.

In conclusion, we show an anti-inflammatory effect of low, physiological doses of C4 on Mtb-induced inflammatory responses. The anti-inflammatory cytokine IL-10 may play a role in mediating the inhibitory effects of C4 on the host immune response to Mtb. Further studies are needed to precisely explore the pathways by which physiological concentrations of C4 exert their anti-inflammatory effects, and to define the mechanism of increased TB sensitivity in type 2 DM patients. Moreover, current research on modulating gut microbiota in DM should include its possible effects on TB.
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**SUPPLEMENTARY MATERIAL**

**Supplementary Figure 1** | Effect of SCFAs, CHX, and aspirin on pH and cell death. (A) PBMCs were incubated with 2-250 µM SCFAs, immediately after which pH levels of culture medium were measured (n = 1). (B) PBMCs were pre-incubated with cycloheximide (CHX) or aspirin for 1 h, prior to 1 h incubation with C4 (50 µM). After 24 h stimulation with Mtb lysate or LPS cells were harvested and stained with Annexin-V / PI-stain to determine levels of cell death. Data are % means ± SEM (n = 3 to 5), representative of 1-2 independent experiments.
Supplementary Figure 2 | PBMCs were pre-incubated with 50 µM C4 (1 h) and stimulated with Mtb lysate or LPS. Cell lysates were harvested at 30 min post stimulation. Phospho-JNK protein levels were determined by western blot using specific antibody (n = 2).

Supplementary Figure 3 | Influence of butyrate on the eicosanoid pathway. (A) COX-2 expression levels in PBMCs pre-incubated with 50 µM C4 for 1 h prior to stimulation with Mtb lysate or LPS for 4 h, as measured by qPCR. Data are single values (n = 10), Wilcoxon signed rank test, representative of 3 independent experiments. (B) To inhibit PGE2, PBMCs were pre-incubated with aspirin for 1 h, prior to 1 h incubation with C4 (50 µM). TNF-α and IL-1β transcript levels were measured by qPCR 4 h after stimulation with Mtb lysate or LPS. Data are means ± SEM (n = 3), Wilcoxon signed rank test, representative of 1 experiment.
Supplementary Figure 4 | Butyrate modulates the immune response to Mtb independently of β-oxidation. PBMCs were pre-incubated with Etomoxir, Ranolazine, Trimetazidine, or Pertussis toxin for 1 h prior to incubation with 50 μM C4 (1 h). After 24 h stimulation with Mtb lysate TNF-α and IL-1β cytokine levels were measured in supernatants by ELISA. Data are means ± SEM (n = 3), Wilcoxon signed rank test, representative of 1 experiment.
Supplementary Figure 5 | Butyrate modulates Mtb-induced cytokine responses. (A) PBMCs were pre-incubated with 50 µM C4 for 1 h prior to stimulation with Mtb lysate or LPS. After 7 days cells were re-stimulated and stained extracellularly for CD4 (CD4-PeCys7) and intracellularly for IL-17 and IL-22 (IL-17-AlexaFluor, IL-22-PE). Data are % means ± SEM (n = 8), Wilcoxon signed rank test, representative of 3 independent experiments. ** p < 0.01 (B) Cytokine levels of IL-1Ra in PBMCs pre-incubated with 50 µM C4 (1 h) and stimulated with Mtb lysate or LPS (24 h) as measured by ELISA. The boxplot represents median with first and third quartiles; the whiskers represent minimum and maximum values. n = 11, Wilcoxon signed rank test, representative of 4 independent experiments. * p < 0.05
PART II
Cellular metabolism of immune cells and host defence to tuberculosis
Chapter 5

Rewiring cellular metabolism via the AKT/mTOR pathway contributes to host defence against Mycobacterium tuberculosis in human and murine cells

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ABSTRACT

Cells in homeostasis metabolise glucose mainly through the tri-carboxylic acid (TCA) cycle and oxidative phosphorylation (OXPHOS), whilst activated cells switch their basal metabolism to aerobic glycolysis. In this study, we examined whether metabolic reprogramming towards aerobic glycolysis is important for the host response to *Mycobacterium tuberculosis* (Mtb). Through transcriptional and metabolite analysis we show that Mtb induces a switch in host cellular metabolism towards aerobic glycolysis in human peripheral blood mononuclear cells (PBMCs). The metabolic switch is TLR2-dependent but NOD2-independent, and is mediated in part through activation of the AKT-mTOR pathway. We show that pharmacological inhibition of the AKT/mTOR pathway inhibits cellular responses to Mtb both in vitro in human peripheral blood mononuclear cells, and in vivo in a model of murine tuberculosis. Our findings reveal a novel regulatory layer of host responses to Mtb which will aid understanding of host susceptibility to Mtb, and which may be exploited for host-directed therapy.
INTRODUCTION

In the absence of HIV co-infection, it is mostly unknown what determines susceptibility to *Mycobacterium tuberculosis* (Mtb), which accounts for almost two million deaths annually.

Recent studies suggest that immune activation and cellular energy metabolism may be relevant since changes in metabolism profoundly influence cell fate and effector functions. For example, it has been shown that changes in glucose metabolism influence T-cell lineage polarisation, innate and adaptive cellular memory and even protein transport and secretion in response to cellular activation [1-4]. Naïve and tolerant cells appear to primarily rely on the tri-carboxylic acid (TCA) cycle and oxidative phosphorylation (OXPHOS) for metabolising glucose and generating stores of ATP necessary for cell survival and function. Upon activation however, a switch towards aerobic glycolysis rewires intracellular glucose metabolism [5]. This permits a significant increase in ATP production and, through the pentose phosphate pathway, provides the nucleotides necessary for cell proliferation [6]. Recent studies by Shi et al. and Gleeson et al. have shown that a shift towards glycolysis is an important component of host defence in murine models of tuberculosis [7, 8]. Taking these studies further, we have investigated which cells, receptors and regulators promote the Mtb-induced switch to glycolysis in humans. We have tested our hypotheses both in in vitro and in vivo experimental models and verified if such metabolic reprogramming also occurs in patients with active pulmonary TB.
MATERIALS AND METHODS

Healthy Volunteers
Peripheral blood mononuclear cells (PBMCs) were isolated from EDTA tubes or buffy coats obtained after informed consent of healthy volunteers (Sanquin Bloodbank, Nijmegen, Netherlands). As donations were anonymous, no tuberculosis skin tests or IFN-γ release assay was performed. The incidence of tuberculosis in the Dutch population is extremely low (1.5/100,000), and Bacillus Calmette-Guérin (BCG) vaccination is not part of the routine vaccination program. Experiments were conducted according to the principles expressed in the Declaration of Helsinki.

Isolation of PBMCs, CD14+ Monocytes and CD3+ T cells
Isolation of PBMCs was performed as described previously [32]. CD14+ monocytes or CD3+ T cells were purified from freshly isolated PBMCs using MACS microbeads by positive selection according to the manufacturer’s instructions (Miltenyi Biotec, Germany).

Stimulation Experiments
For stimulation experiments, 5x10^6 PBMCs/mL or 1x10^6 monocytes/mL were stimulated with RPMI, 1 μg/mL Mycobacterium tuberculosis strain H37Rv (Mtb) lysate, 10 μg/mL Pam3Cys (EMC Microcollections, Germany) and/or 10 μg/mL muramyl dipeptide (MDP; Sigma) in the presence or absence of 1 nM or 10 nM rapamycin (LC-laboratories), 100 nM Torin (Tocris), 50 μM or 500 μM sodium L-ascorbate (Sigma), 50 μM or 500 μM AICAR (Brunschwig chemie), 100 nM wortmannin (Cayla Invivogen), or 20 ng/mL or 100 ng/mL of TLR4 inhibitory ligand double-extracted Bartonella quintana LPS for 24 h or with 10% pooled human serum for 7 d. PBMCs from patients with the homozygous carriage of a frameshift in Nod2 due to an insertion of cysteine at position 1007 (1007finsC) (rs2066847) were stimulated with RPMI, MDP or Mtb for 1 or 7 d. Culture supernatants were collected and stored at −20°C. For Western blots, 5x10^6 PBMCs were stimulated with RPMI, 1 μg/mL Mtb lysate or 50 ng/mL GM-CSF in the presence or absence of the aforementioned inhibitors. Cytokine measurements from cell culture supernatants were performed by ELISA namely interleukin (IL)-1β, tumour necrosis factor (TNF)-α, IL-17A, IL-22 (R&D Systems, Minneapolis, MN); and IL-6, interferon gamma (IFN-γ) and IL-10 (Sanquin, Amsterdam, Netherlands) were measured.
**Animal Experiments**

TLR2 knockout mice were kindly provided by Prof S. Akira (Department of Host Defense, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan) and were fully backcrossed to the C57BL/6 background. Age- and sex-matched control C57BL/6 mice were obtained from Charles River Wiga (Sulzfeld, Germany).

Wild-type, NOD1, NOD2 and NOD1/NOD2 double knockout mice were bred and maintained in the St. Jude Children’s Research Hospital (Memphis, Tennessee). After dissection of mouse legs, the bone marrow was flushed out using sterile PBS and cells obtained were differentiated over a period of 7 d at 37°C, 5% CO₂ in Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Invitrogen, Carlsbad, CA, USA) supplemented with 30% L929 medium, 10% heat-inactivated filtered foetal bovine serum (Invitrogen, Carlsbad, CA, USA), 1% nonessential amino acids (Life Technologies), 100 U/mL penicillin and 100 mg/mL streptomycin. On day 6 BMDMs were washed, counted and seeded in 96-well plates at a concentration of 1×10⁵ cells/well or in 6-well plates at 1×10⁶ cells/well for Western blotting. Cells were left to rest overnight at 37°C, after which they were stimulated with medium, namely 10 μg/mL Pam3Cys, 1 μg/mL Mtb or 100 nM insulin. Experiments were approved by the Ethics Committee on Animal Experimentation of the Radboud University Medical Center and protocols were approved by the St. Jude Children’s Research Hospital Committee on the Use and Care of Animals.

Female 8- to 10-week-old C57BL/6 mice were kept under pathogen-free conditions at the Max Planck Institute for Infection Biology in Berlin, Germany (ethical approval of the Berlin Office for Health and Social Affairs reference number G 0179/12). Mtb strain H37Rv was grown in Middlebrook 7H9 broth (BD Biosciences) supplemented with 0.2% glycerol, 0.05% Tween 80 and 10% ADC enrichment (BD Biosciences) until it reached a mid-logarithmic growth phase and then stored at –80°C. Animals were aerosol-infected with 200 CFUs using a Glas-Col inhalation exposure system. Rapamycin (Sigma) or vehicle (PBS/100% EtOH) was injected the day before aerosol infection and thereafter daily for 28 days. Splenocytes were isolated from single-cell suspensions prepared by mechanical dissociation through a 70-µm nylon mesh in RPMI 1640 medium with 10% foetal calf serum (Gibco). Splenocytes were seeded in a 24-well-plate at a concentration of 2.5×10⁶ cells/mL and stimulated with 1 mg/mL of Mtb lysate, 1 mg/mL of PPD (Staten Serum Institut, Copenhagen), 10 mg/mL PHA (Sigma) or 10 ng/mL of LPS (from E. coli 055:B5, Sigma). After 6 d supernatants were collected and stored at –20°C. Cytokines were analysed using a bead-based assay (Bio-Rad).
Metabolite Measurements
Lactate was measured from cell culture supernatants using a coupled enzymatic assay in which lactate was oxidised and the resulting H$_2$O$_2$ was coupled to the conversion of Amplex® Red reagent to fluorescent resorufin by HRP (horseradish peroxidase) [33]. Measurement of the NAD$^+/NADH$ redox ratio was adapted from Zhu et al [34]. Glucose consumption was measured according to the manufacturer’s instructions using the Amplex® Red Glucose/ Glucose Oxidase Assay Kit (Life Technologies).

Real-time analysis of the extracellular acidification rate (ECAR) and the oxygen consumption rate (OCR) on CD14+ monocytes was performed using an XF-96 Extracellular Flux Analyzer (Seahorse Bioscience). Briefly, monocytes were plated in XF-96 cell culture plates (2 x 10$^5$ monocytes/well) in the presence of RPMI or Mtb lysate for 24 h in 10% human pooled serum. The monocytes were washed and analysed in XF Base Medium (unbuffered DMEM with 25mM glucose and 2 mM L-glutamine, pH was adjusted to 7.4). Basal OCR and ECAR of the monocytes was measured every 5 min for 20 min in total.

Cell Viability Assessment
The percentage of cells that underwent early or late apoptosis was determined by labelling with annexin V–fluorescein isothiocyanate (FITC, Biovision) and staining with propidium iodide (PI, Sigma Aldrich), according to the manufacturer’s instructions. Briefly, stimulated PBMCs were re-suspended in 200 μL of RPMI and incubated on ice in the dark with 1 μL of Annexin V-FITC for 15 min followed by a 5 min incubation with 1.5 μL of PI. The relative level of apoptotic cells was detected by flow cytometry within 1 h, using a FC500 flow cytometer (Beckman Coulter) and data were analysed using Kaluza 1.3 software (Beckman Coulter).

Western Blotting
Western blotting was carried out using a Trans Turbo Blot system (Bio-Rad) according to the manufacturer’s instructions. 5x10$^6$ PBMCs or 1 x 10$^6$ CD14$^+$ monocytes or 1 x 10$^6$ CD3$^+$ T cells were lysed in 100 μL lysis buffer. The cell homogenate was frozen, thawed and processed for Western blot analysis. Equal amounts of protein were resolved by SDS-PAGE on 4-15% polyacrylamide gels (Bio-Rad). Separated proteins were transferred to PVDF (Bio-Rad) membranes. According to the manufacturer’s instructions, the membrane was blocked for 1 h and then incubated overnight with a primary antibody at a dilution of 1:1000 in 5% (w/v) bovine serum albumin (BSA, Sigma) or milk in TBS-Tween buffer (TBS-T). After overnight incubation, blots were washed in TBS-T 3 times and incubated with HRP-conjugated anti-rabbit antibody (1:5000; Sigma) in 5% (w/v) milk in TBS-T for 1 h at RT. After washing the blots were developed with ECL (Bio-Rad) according to the manufacturer’s
networks. The primary antibodies used were rabbit anti-actin (Sigma), mAB phospho-p70 S6 Kinase (Thr389) (Cell Signalling), phospho-AKT (Cell Signalling), and phospho-4EBP1 (Cell Signalling).

**Transcriptome Analyses**

We obtained previously published transcriptome data for our analysis (GSE42606) [35]. Briefly, PBMCs were stimulated with RPMI or Mtb (1μg/mL) for 24 h at 37°C and 5% CO₂. Total RNA was extracted in 800μL of TRizol reagent (Invitrogen). Global gene expression was profiled using an Illumina Human HT-12 Expression BeadChip according to the manufacturer’s instructions. Image analysis, bead-level processing and quantile normalisation of array data were performed using the Illumina LIMS platform, BeadStudio. The quantile normalised expression data for glycolysis and TCA cycle genes (derived from KEGG pathway) were extracted to perform differential expression analysis between RPMI and Mtb stimulations.

**Human Gene Expression Analysis**

Publicly available micro-array data (Illumina Human HT-12 V3 BeadChip) from cohorts of patients with active and latent tuberculosis and uninfected controls in the UK and South-Africa [9] were obtained from the Gene Expression Omnibus (GEO) under accession number GSE19491. We restricted our analysis to the expression data form whole blood (collected in Tempus tubes, Applied Biosystems) from the UK test cohort and the South-African validation cohort. Genes implicated in glycolysis (path:hsa00010) and TCA cycle (path:hsa00020) pathways were extracted from KEGG, putative genes were removed and the remaining genes were converted into Illumina probe numbers using DAVID [36, 37]. Probes that had a negative expression for one of the samples were excluded.

**Statistical Analysis**

Principal component analysis (PCA) was performed on log-transformed data using singular value decomposition separately for the glycolysis and TCA cycle pathways in Python. Scores of the samples projected on principal component (PC) 1 for glycolysis were plotted against the scores on PC1 for the TCA cycle, to show the maximum combined variance of both pathways in one graph. Additionally, binary regression analysis was performed on the scores projected on PC1 for both pathways with LTBI (0) versus PTB (1) for the South-African cohort in SPSS 21.

The heat-maps show differences in expression based on log₂ transformed data from the different groups. Statistical testing of the in vitro data set was performed on the mean expression between the unstimulated (RPMI) and stimulated (Mtb) samples using a Wilcoxon signed-rank test for parametric data; adjusted p-values less than 0.05 were considered
significant. LTBI versus PTB patients were compared using Mann-Whitney U tests. Colour
coding was based on the minimum and maximum for each of the comparisons. In the
legend, changes were transformed back for interpretability. Genes that showed a differential
expression in any of the datasets are presented in the figure.

Differences in cytokine production were analysed using the Wilcoxon signed-rank test for
non-parametric distributions. Data were considered statistically significant at a p-value <
0.05. Data are shown as cumulative results of levels obtained in all volunteers (means ±
SEM).
RESULTS

Transcriptome analysis of whole blood from patients with pulmonary TB and human PBMCs stimulated with Mtb reveals a shift towards glycolysis. Based on expression of genes coding for glycolysis or TCA cycle pathways, pulmonary TB patients (PTB) could be clearly separated from individuals with latent tuberculosis infection (LTBI) or healthy controls (CON) using principal component analysis (PCA) of publically sourced human expression data [9] (Figure 1A and 1B). Regression analysis on the first component of both groups showed that both pathways were significantly different between LTBI and PTB (p < 0.001 for both) groups. Individuals on anti-tuberculosis treatment were spread throughout the plot with untreated cases closer to PTB and those on 12 months of treatment interspersed with the LTBI and CON groups. Analysis of individual genes in the PTB versus CON groups clearly showed an up-regulation of glycolysis genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and hexokinase-3 (HK3) (Figure 1C and 1D), which returned towards normal following treatment. The opposite was observed for the TCA cycle gene isocitrate dehydrogenase 3 (NAD+ beta) (IDHB3B) (Figure 1E).

We next examined expression of similar metabolic genes in an in vitro model. Similar to the in vivo dataset described above, Mtb-stimulated PBMCs also displayed a strong up-regulation of some genes involved in glycolysis and down-regulation of some involved in the TCA cycle (Figure 1F).

Based on the in vitro and in vivo data analyses, an intracellular map was generated to reflect the switch to aerobic glycolysis induced upon Mtb stimulation (Figure 1J). The general metabolic shift is further supported by altered expression of individual genes. SLC16A3 (solute carrier family 16 monocarboxylate transport MCT, member 3), which encodes a lactate transporter, was significantly upregulated in PTB patients and returned to normal levels upon treatment (Supporting Information Figure 1A). Conversely, sirtuin 5 (SIRT5), a protein whose activity is directly linked to the energy status of the cell via the cellular NAD+/NADH ratio, was down-regulated in PTB patients and normalised upon treatment (Supporting Information Figure 1B).
M. tuberculosis induces glycolysis

Figure 1 | Transcriptional regulation of glucose metabolism during active TB disease and in PBMCs stimulated with Mtb. (A, B) Principal component analysis (PCA) of the glycolysis and TCA cycle whole blood gene signatures of microarray data from publically available cohorts from (A) the United Kingdom (UK) and (B) South Africa (SA), previously published by Berry et al (Series GSE19444) [9]. Data for each sample, including active pulmonary TB (PTB), LTBI (TST-positive) and CON (TST-negative) individuals were plotted along PC1 for glycolysis versus PC1 for the TCA cycle. For the UK cohort, PC1 accounted for 37% of the total variation for both glycolysis and the TCA cycle. For the South African cohort PC1 accounted for 44% and 34% of the variance for glycolysis and the TCA cycle respectively. (C-E) Individual and mean whole blood gene expression for (C) GAPDH, (D) HK3 and (E) IDH3B in controls and PTB patients on 0 m, 2 m or 12 m of TB treatment (Series GSE19435). Microarray data derived from Berry et al [9]. Symbols represent individual samples and data are shown as means ± SEM; means were compared using the Mann-Whitney U test. *p<0.05, **p<0.01, ***p<0.001. (F) Heatmap of gene expression pattern of glycolysis or TCA cycle genes in in vitro-stimulated PBMCs and in vivo whole blood (UK and SA publically available cohorts). All data was log-2 transformed. The in vitro Mtb stimulations were normalised to RPMI by subtracting means whereas the in vivo PTB cohort was normalised to the corresponding LTBI cohort. P-values were considered significant when less than 0.05 as determined by the Wilcoxon signed-rank test for the in vitro data set and by the Mann-Whitney U test for the in vivo data sets. Red represents a significant up regulation, blue a significant down regulation and grey no difference. (G) Schematic representation of up-regulated (in red) and down-regulated (blue) genes in the glycolysis and TCA cycle pathways as determined by microarray analysis of Mtb-stimulated PBMCs.

Mtb drives cellular commitment to aerobic glycolysis in human monocytes and macrophages. Monocytes, macrophages, dendritic cells, T helper (T<h>1</h>) and T<h>17</h> cells use glucose as a substrate for energy production [10]. To determine the commitment to glycolytic metabolism in stimulated monocytes, we analysed the extracellular acidification rate (ECAR) as an indicator of the glycolytic rate 24 h post-stimulation. There was a significant increase in all three baseline measurements and ECAR levels were approximately 3.3 fold higher in Mtb stimulated cells (Figure 2A). This increase in ECAR was responsible for a decrease in the OCR/ECAR ratio, as previously described for LPS [11] (Supporting Information Figure 2A), despite a slightly higher oxygen consumption rate (OCR) (Figure 2B). Similarly, macrophages infected with live Mtb or stimulated with Mtb lysate produced increased levels of lactate, one of the hallmarks of glycolysis (Figure 2C). Additionally, PBMCs challenged with Mtb showed increased glucose consumption (Figure 2D) and lactate production (Figure 2E). Generation of lactate requires the oxidation of NADH to co-factor NAD⁺ by lactate dehydrogenase. Accordingly, we observed a significant increase in the ratio of NAD⁺/NADH in Mtb stimulated PBMCs (Figure 2F). Conversely, resting PBMCs displayed a lower NAD⁺/NADH ratio reflecting cellular metabolism mostly via the TCA cycle rather than glycolysis (Figure 2F).
**Figure 2 | Physiology of the PBMC metabolic response to Mtb stimulation.** (A, B) CD14+ monocytes were stimulated for 24 h with Mtb and (A) ECAR and (B) OCR rates were determined using the Seahorse metabolic analyser. Three baseline measurements were determined. Data are shown as means ± SEM (n = 7). (C) Lactate production from macrophages stimulated with live H37Rv (10:1 MOI) was measured by a fluorescent coupled enzymatic assay. Data are shown as means ± SEM of n = 4, pooled from two independent experiments. (D-F) PBMCs were stimulated with Mtb lysate and the kinetics of (D) glucose consumption, (E) lactate production and (F) the intracellular NAD+/(NADH) ratios from days 1, 3 and 7 was measured by metabolite specific coupled enzymatic assays. Data are shown as means ± SEM of n = 6 to 8, pooled from three independent experiments. Means were compared using the Wilcoxon signed-rank test, *p<0.05).

**Mtb activates AKT and mTOR in human PBMCs.** As mammalian target of rapamycin (mTOR) is the master regulator of cell growth, proliferation and metabolism, [12] and AKT-mediated mTOR activation induces glucose metabolism, [13, 14] we assessed whether Mtb activated the AKT-mTOR pathway (Figure 3A). Indeed, sensing of the microorganism induced AKT activation, which was inhibited by the phosphatidylinositol-3 kinase (PI-3K) inhibitor wortmannin (Figure 3B). In addition, p70-S6K and 4E-BP1, two canonical downstream targets of mTOR, were activated upon Mtb stimulation. Purification of CD14+ monocytes and CD3+ T cells from stimulated PBMCs revealed that Mtb induced mTOR activation in monocytes but not T-cells (Figure 3C).
Figure 3 | Induction of glycolysis in human PBMCs is mediated by the AKT-mTOR Pathway. (A-D) PBMCs were stimulated with RPMI or Mtb in a time-dependent manner in the presence or absence of DMSO (vehicle control), wortmannin (PI3K/AKT inhibitor) or rapamycin (mTOR inhibitor). (C) CD14+ and CD3+ T cells were separated from PBMCs stimulated for 2 h with Mtb. Where indicated, GM-CSF stimulation was included as a positive control. (A, B) AKT, (C, D) p70S6K and 4E-BP1 phosphorylation and actin levels were determined by western blot using specific antibodies. (A, B) Cell lysates were harvested at 15, 30, 60 and 120 min post-stimulation. (C, D) Cell lysates were harvested at 30, 60, 120 and 240 min post-stimulation. Representative blots from two of four donors are shown. (E-G) PBMCs were pre-incubated with 10 nM rapamycin, 100 nM torin or 100 nM wortmannin for 1 h prior to stimulation with Mtb lysate. Data are shown as means ± SEM of n = 9, pooled from three independent experiments. Means were compared using the Wilcoxon signed-rank test (*p<0.05, **p<0.01).

Activation of these mTOR targets was inhibited by rapamycin, an mTOR inhibitor, in both PBMCs (Figure 3D) and CD14+ monocytes (Supporting Information Figure 3A).

Additionally, rapamycin, torin and wortmannin significantly decreased lactate release induced by Mtb, further demonstrating the involvement of the mTOR/AKT pathway in induction of glycolysis by Mtb (Figures 3E, 3F and 3G).
mTOR regulates T\(_H\) cell-derived cytokines from PBMCs in response to Mtb.

T-lymphocytes form a crucial component of the host defence against Mtb. Direct inhibition of glycolysis by 2-deoxy-glucose (2DG) led to a dose-dependent decrease in the production of the T\(_H\) cytokines IFN-\(\gamma\), IL-17 and IL-22 from PBMCs in response to Mtb (Figure 4A). Direct inhibition of mTOR by rapamycin or torin or indirectly by AICAR (an AMPK activator; Figures...
4B and 4C), and inhibition of mTOR-dependent HIF-1α activation by ascorbate also resulted in reduced T
α-derived cytokine production (Figure 4D). IL-10 production in response to Mtb was also significantly inhibited upon mTOR inhibition (Supporting Information Figures 4A, 4B and 4D). Production of monocyte-derived TNF-α, IL-6 and IL-1β was mostly reduced upon inhibition of glycolysis with 2DG (Supporting Information Figure 4A). Interestingly, production of monocyte-derived cytokines remained mostly unchanged in the presence of rapamycin, torin and ascorbate (Supporting Information Figures 4B, 4C and 4D). These inhibitors did not affect cell survival at the time points used for the stimulation experiments (Supporting Information Figures 5A, 5B, 5C, 5D and 5E).

**Induction of glycolysis by Mtb is TLR2-dependent but NOD2-independent**

We next examined which of the main pattern recognition receptors (PRRs) that mediate recognition of Mtb, [15] are necessary for a metabolic switch towards aerobic glycolysis. PBMCs isolated from patients with a complete deficiency in NOD2 showed decreased cytokine production in response to Mtb (Figure 5A), but no decrease in lactate production (Figure 5B). In addition, bone marrow-derived macrophages (BMDMs) from NOD2 knockout (NOD2
−/−) and NOD1/NOD2 double knockout (NOD1
−/−/NOD2
−/−) mice stimulated with Mtb showed decreased KC and IL-6 production. However, as with the NOD2 deficient patients, no change in lactate production was observed and no major differences were apparent after stimulation of BMDMs from NOD1 knockout (NOD1
−/−) mice either (Figure 5C and 5D).

We then examined possible synergy with regard to lactate production between NOD2 and the TLRs involved in recognition of Mtb. As expected, cytokine production of PBMCs stimulated with LPS (TLR4 ligand), Pam3Cys (P3C, TLR2 ligand) or Mtb increased in the presence of muramyl dipeptide (MDP, an NOD2 ligand), whilst MDP by itself did not induce cytokine production (Figures 6A and 6B). On the other hand, MDP showed no synergistic effect for lactate production upon stimulation with the various other TLR ligands (Figure 6C).
Figure 5 | Role of NOD2 in the induction of glycolysis. (A, B) PBMCs from NOD2-deficient patients (n=2) and healthy volunteers (n=4) were stimulated with RPMI, MDP and Mtb for 24 h and 7 d. The levels of (A) indicated cytokines or (B) lactate production were measured from culture supernatants by ELISA and an enzymatic couple assay respectively. Data are shown as means ± SEM of the indicated number of donor samples and are from a single experiment. (C, D) Bone marrow-derived macrophages (BMDMs) from wild type (WT), NOD1 knockout (-/-), NOD2 knockout (-/-) and NOD 1/2 double knockout (-/-) mice were stimulated with RPMI or 1 μg/mL Mtb lysate for 24 h (n=2). The levels of (C) IL-6 and KC and (D) lactate production were measured as described above. Data are shown as means ± SEM of n = 2 from a single experiment.
To investigate the role of TLR4 in the induction of aerobic glycolysis by Mtb, we blocked TLR4 by pre-incubation of PBMCs with *Bartonella quintana* LPS, a potent natural antagonist of TLR4 (Popa et al., 2007), before stimulating with Mtb or *E. coli* LPS. Although a potent decrease in IL-6 and lactate levels was observed for LPS, indicating effective blocking of TLR4, no effect of the TLR4 blockade on Mtb-induced lactate was seen (Figures 6D and 6E).

To investigate the role of TLR2, we stimulated BMDMs and peritoneal macrophages from TLR2 knockout (TLR2⁻/⁻) mice with Mtb and Pam3Cys. Production of KC (IL-8) and lactate was potently decreased in TLR2⁻/⁻ BMDMs compared to wild-type controls (Figures 6F, 6G and 6H). In addition, stimulation of cells isolated from TLR2⁻/⁻ mice with Mtb led to a decrease in AKT activation compared to controls, whilst the induction of AKT with insulin, in a TLR independent manner, was unaffected (Figure 6I). Collectively, these data implicate TLR2, and not TLR4 or NOD2, as the major pattern recognition receptor that induces AKT/mTOR pathway activation and thus mediates the switch to aerobic glycolysis upon Mtb stimulation.
Figure 6 | Stimulation via TLR2 initiates rewiring of cellular metabolism in mouse macrophages. (A-C) PBMCs from healthy volunteers (n=6) were stimulated with RPMI, MDP +/- LPS, Pam3Cys (P3C) or Mtb lysate for 24 h. The levels of (A) IL-1β, (B) IL-6 and (C) lactate in cell culture supernatants were measured as described above. Data are shown as means ± SEM of n = 6 pooled from 2 experiments. Means were compared using the Wilcoxon signed-rank (*p<0.05). (D, E) PBMCs were pre-incubated with TLR4 antagonist Bartonella quintana LPS (20 and 100 ng/mL) prior to stimulation with RPMI, Mtb lysate or LPS. The levels of (D) IL-6 production and (E) lactate in culture supernatants were determined by ELISA and a coupled enzymatic assay respectively. Data are shown as means ± SEM of n = 6 to 8 pooled from three experiments. Means were compared using the Wilcoxon signed-rank test (*p<0.05). (F-H) BMDMs and peritoneal macrophages (Mfs) from TLR2 knockout (TLR2-/-) mice were stimulated with RPMI, P3C (positive control) and Mtb lysate. The levels of (F) KC (IL-8) and (G, H) lactate in culture supernatants were measured by ELISA and a coupled enzymatic assay. Data are shown as means ± SEM of n =3-6 pooled from two experiments. Means were compared using the Wilcoxon signed-rank test. (I) Levels of AKT activation from TLR2-/- BMDMs stimulated with RPMI, insulin (control; INS) and Mtb lysate (Mtb) was determined by western blot. Actin was used as loading control. One of two representative blots is shown.
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mTOR inhibition in an in vivo murine experimental model of TB

We then examined the in vivo effects of mTOR inhibition on Mtb-induced cytokine responses. C57BL/6 mice were injected with rapamycin or control vehicle one day before aerosol infection with Mtb and thereafter daily for 28 d (Figure 7A). At 28 d post infection, ex vivo stimulations of splenocytes with Mtb lysate, PPD, PHA and LPS revealed a potent reduction in the capacity of the rapamycin-treated mice to respond to both Mtb lysate (Figure 7B) and non-specific stimuli like PPD, PHA and LPS (Supporting Information Figures 6A - 6V). Notably, the production of the T_H1-derived cytokines IFN-γ and IL-17 was inhibited by rapamycin (Figure 7B), as previously observed in vitro. Systemic inhibition of mTOR also led to the inhibition of pro-inflammatory cytokines IL-12 p70 and TNF-α (Figure 7B). It is highly likely that due to the anti-mycobacterial effects of rapamycin [16], rapamycin injections did not affect Mtb outgrowth (Supporting Information Figure 6W).

Figure 7 | In vivo effects of mTOR inhibition. (A, B) C57/BL6 mice were treated with rapamycin or vehicle (mixed PBS/100% EtOH) from 1 d prior to aerosol infection with Mtb until 28 d post-infection. Mice were euthanised and splenocytes were harvested and re-stimulated with Mtb lysate (1 mg/mL) for 6 d, after which (B) a bead-based immunoassay for mTNF-α, mIL-12p70, mIL-17 and mIFN-γ was performed. Data are shown as means ± SEM of n =6 samples from a single experiment. Means were compared using the Mann–Whitney U test (*p<0.05).


DISCUSSION

A metabolic switch to aerobic glycolysis, also known as the Warburg effect, was first described by Otto Warburg in cancer cells [17]. In recent years an increasing body of literature has shown that a similar pattern of metabolic rewiring is important for differentiation of effector \( \text{Th} \) lymphocytes [5], activation of macrophages and dendritic cells [1] and more recently for Mtb infection [7, 8]. Expanding on this, we used transcriptome data to demonstrate that this switch takes place in patients with active TB disease and we thereafter performed functional experiments to identify the molecular mechanisms governing this switch to glycolysis. Specifically, we show that the switch to glycolysis in cells that encounter Mtb relies on TLR2 recognition and is in part dependent on the intracellular AKT-mTOR axis.

Aerobic glycolysis is an ancient process for generating ATP seen even in early single-cell eukaryotes such as yeast. Although primitive in comparison to the TCA cycle and OXPHOS, aerobic glycolysis rapidly facilitates the high bio-energetic needs of cells responding to intruding pathogens. We show that this metabolic switch is also necessary for cells to mount an efficient response to Mtb.

The mTOR regulatory complex has been highly conserved throughout evolution. It integrates extracellular and intracellular signals to regulate cell growth, metabolism, proliferation and survival. The mTOR protein can form two distinct multi-protein complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). Mtb stimulation increased mTORC1 activity as measured by an increase in phosphorylation of its targets 4E-BP1 and p70-S6K1. Additionally, rapamycin inhibits mTORC1 but cannot physically interact or acutely inhibit mTORC2. This suggests that the Mtb-induced switch to aerobic glycolysis is in part due to mTORC1 activation. The involvement of mTORC1 is further supported by studies showing that naive T helper cells preserve their ability to differentiate into \( \text{Th}1 \) and \( \text{Th}17 \) cells in the absence of mTORC2 signalling, but not in the absence of mTORC1 signalling [18]. Mechanistically it would be highly interesting to determine whether mTORC2-deficient cells could commit to glycolysis. Of note, blockade by rapamycin, torin and wortmannin individually does not completely ablate lactate production suggesting redundancy of these regulators, regulation by other pathways or incomplete pharmacological inhibition. Dissecting if the mTOR/AKT pathway is the sole regulator of glycolysis would therefore be of future interest.

NOD2 and TLR receptors are major pattern recognition receptors for Mtb, with synergistic effects on Mtb-mediated cytokine production [19, 20]. It is thus interesting that rewiring of cellular glucose metabolism is strictly TLR2-dependent (but NOD2-independent), and that the NOD2 agonist MDP does not potentiate TLR2-induced lactate production.
The discrepancy between the effects of NOD2 on cytokine responses and induction of glycolysis suggests the presence of distinct intracellular pathways responsible for these two processes. Whilst NOD2 stimulation is known to induce MAPK activation and NF-kB translocation leading to cytokine induction [21], activation of the AKT/mTOR pathway is not demonstrated. In contrast, TLRs induce both MAPK, NF-kB and the AKT/mTOR pathway [22, 23]. Moreover, activation of AKT inhibits the NOD2-mediated NF-kB pathway [24], while NOD1 stimulation (that activates similar intracellular pathways) inhibits AKT phosphorylation induced by insulin [25]. These data strongly suggest a fundamental difference between TLRs and NOD2 for activation of AKT/mTOR pathway, with NOD2 unable to induce AKT activation and thus metabolic reprogramming. Future studies that compare this requirement for TLR2 in other infectious stimuli, such as Gram positive or negative bacteria, would be of interest.

T_{H1} cells exert different effector functions and thus it is not surprising that they exhibit distinct metabolic programs. T effector cells primarily rely on mTOR-driven aerobic glycolysis for energy, whilst regulatory T cells rely on lipid oxidation and mitochondrial respiration mediated by AMPK [1]. Mtb induced T_{H17} cells appear to be more dependent on aerobic glycolysis than T_{H1} cells, possibly due to the upregulation of HIF-1α [26]. HIF-1α is a hypoxia-induced transcription factor that directly controls gene expression of enzymes in the glycolysis pathway [27]. The role of HIF-1α in the generation of IFN-γ-producing T_{H1} cells is unclear, as either enhanced or no differences in IFN-γ production have been observed in experimental models lacking HIF-1α [28, 29]. The use of ascorbate, an HIF-1α inhibitor, emulates these observations, as a significant decrease in IL-17 but not IFN-γ production was found in PMBCs.

In vitro inhibition of mTOR with rapamycin or torin led to either no differences or an increase in TNF-α, IL-6 and IL-1β production, in line with literature showing that mTOR inhibits pro-inflammatory cytokine production via negative regulation of NF-kB, whilst inhibiting T cell proliferation [30]. Nonetheless, in our in vivo Mtb infection model, prolonged exposure to rapamycin inhibited overall cytokine responses to Mtb including pro-inflammatory cytokines such as IL-12 and TNF-α. Likewise, 2-DG, a direct competitive inhibitor of glycolysis, inhibited both monocyte and T cell-derived cytokines. As mentioned above, the HIF-1α inhibitor ascorbate did not influence monocyte-derived cytokines, in contrast to a previous study showing HIF-1α dependent LPS-mediated IL-1β production in murine macrophages [31]. This may be explained by the differences in required doses of ascorbate, or discrepancies between human and mouse glucose metabolic processes.

In this study we take a step further in dissecting the role of leukocyte energy metabolism in the immune responses to Mtb in vitro and in vivo. Recognition of the fact that metabolic rewiring towards glycolysis is crucial for host immunity leads to the hypothesis that
perturbations in leukocyte metabolism could be detrimental for the host. For instance, impaired glucose metabolism in patients with diabetes leading to reduced capacity to mount the necessary metabolic changes to respond to Mtb may contribute significantly to their increase in TB susceptibility.

In conclusion, our study has cemented glycolysis as a fundamental process that underpins the cellular circuitry needed to mount effective host responses to Mtb and promotes it as a therapeutic target in TB. In particular, pharmacologic manipulation of enzymes in these pathways may generate more robust and effective immune responses and thus open up new avenues for host-directed therapies for tuberculosis.

AUTHOR CONTRIBUTIONS
E.L. designed and performed experiments and wrote the manuscript. M.B.B, A.A., L.B. and M.S.G. performed experiments. V.K., A.L. and X.W. performed data analyses. D.J., T.D.K. and T.H.M.O provided reagents and wrote the paper. S.C.C, L.A.B.J, R.S., C.W, S.H.E.K. discussed the data and wrote the manuscript. R.C and M.G.N discussed data, designed experiments and wrote the manuscript.

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**SUPPLEMENTARY MATERIAL**

**Supporting Information Figure 1 | Transcriptional Regulation of Glucose Metabolism.** (A-B) Data are represented as mean scatter plots for gene expression of MCT4 and Sirtuin 5 in LTBI and PTB individuals in the South African validation cohort of Berry et al. [13] and in controls and PTB patients on 0 m, 2 m or 12 m of treatment with anti-tubercular drugs. Symbols represent individual samples and data are shown as means. Means were compared using the Mann-Whitney U test. *p<0.05, **p<0.01, ***p<0.001.

**Supporting Information Figure 2 | Physiology of the Metabolic Response to Mtb Stimulation.** (A) CD14+ monocytes were stimulated for 24 h with Mtb lysate (n=6) and LPS (n=4) and the resulting ECAR and OCR rates were determined using the Seahorse metabolic analyser. Three baseline measurements were determined. Data are shown as means ± SEM of n = 4 to 6 from a single experiment. Means were compared using the Wilcoxon signed-rank test (*p<0.05).
Supporting Information Figure 3 | Induction of Glycolysis is Mediated by the AKT-mTOR Pathway. (A) CD14+ monocytes pre-incubated with DMSO (vehicle control) or rapamycin (mTOR inhibitor) were stimulated with RPMI (R) or Mtb lysate (T) for 2 h. Cell lysates were analysed by Western blot for phosphorylation of p70-S6K and actin levels using specific antibodies. Blots from two donors are shown.
Supporting Information Figure 4 | Regulation of Monocyte-Derived Cytokines by Aerobic Glycolysis. (A-D) PBMCs were pre-incubated with DMSO (vehicle control) or (A) 1 mM or 5 mM 2DG, (B) 1 nM or 10 nM rapamycin, 100 nM torin, (C) 500 μM AICAR or (D) 50 μM or 500 μM ascorbate or for 1 h prior to stimulation with Mtb lysate. TNF-α, IL-1β, IL-6 and IL-10 levels were measured from culture supernatants by ELISA. Data are shown as means ± SEM of n = 6 to 9 pooled from three independent experiments. Means were compared using the Wilcoxon signed-rank test (*p<0.05, **p<0.01).
Supporting Information Figure 5 | Effects of Metabolic Pathway Inhibitors on Cell Death.

(A–E) PBMCs were pre-incubated with DMSO (vehicle control) or (A) 1 mM or 5 mM 2DG, (B) 1 nM or 10 nM rapamycin, 100 nM torin, (C) 500 μM AICAR or (D) 50 μM or 500 μM ascorbate or for 1 h prior to stimulation with Mtb lysate for 1, 3 or 7 d after which the cells were harvested and stained with an Annexin-V / PI stain to determine levels of cell death. Data are shown as means ± SEM of n = 4 to 6 pooled from three independent experiments. Means were compared using the Wilcoxon signed-rank test.
Supporting Information Figure 6 | In vivo Effects of Rapamycin on Ex vivo Splenocyte Restimulation. (A–W) C57/BL6 mice were treated with rapamycin or vehicle (mixed PBS/100% EtOH) from 1 day prior to aerosol infection until 28 d post-infection. Mice were euthanized. Splenocytes were harvested and re-stimulated with Mtb lysate, PPD, PHA or LPS for 6 d after which a bead-based immunoassay was performed (A–U). Lung homogenates were harvested and CFUs plated and counted after three weeks (W). Data are shown as means ± SEM of n =6 samples from a single experiment. Means were compared using the Mann–Whitney U test (*p<0.05).
Chapter 6

Microbial stimulation of different Toll-like receptor signaling pathways induces diverse metabolic programs in human monocytes

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ABSTRACT

Microbial stimuli such as lipopolysaccharide (LPS) induce robust metabolic rewiring in immune cells, known as the Warburg effect. It is unknown whether this increase in glycolysis but decrease in oxidative phosphorylation (OXPHOS) is a general characteristic of monocytes that have encountered a pathogen. Using CD14+ monocytes from healthy donors we demonstrate that most microbial stimuli increase glycolysis, but only stimulation of Toll-like receptor (TLR) 4 with LPS leads to a decrease in OXPHOS. Instead, activation of other TLRs, such as TLR2 activation by Pam3CysSK4 (P3C), increases oxygen consumption and mitochondrial enzyme activity. Transcriptome and metabolome analysis of monocytes stimulated with P3C versus LPS confirmed the divergent metabolic responses between both stimuli and revealed significant differences in the TCA cycle, OXPHOS and lipid metabolism pathways upon stimulation of monocytes with P3C versus LPS. At a functional level, pharmacological inhibition of complex I of the mitochondrial electron transport chain diminished cytokine production and phagocytosis in P3C-, but not LPS-stimulated monocytes. To summarise, unlike LPS, complex microbial stimuli and the TLR2 ligand P3C induce a specific pattern of metabolic rewiring that involves up-regulation of both glycolysis and OXPHOS which enable activation of host defence mechanisms like cytokine production and phagocytosis.
INTRODUCTION

Otto Warburg first described the concept of metabolic rewiring in tumour cells in the 1930s, and showed that differentiated cells rely on mitochondrial oxidative phosphorylation (OXPHOS) to generate ATP whereas tumour cells switch to aerobic glycolysis under normoxic conditions. In recent years immunologists have discovered that this metabolic shift also underpins the activation and function of immune cells. T-helper cells such as activated T\textsubscript{H}1 and T\textsubscript{H}17 cells are characterised by active glycolysis, whereas quiescent T-cells like regulatory and memory T cells rely on OXPHOS and fatty acid oxidation (FAO) for their ATP production. Similar parallels can be drawn between polarized macrophages, where pro-inflammatory M1 macrophages are highly glycolytic but anti-inflammatory M2 macrophages rely mainly on OXPHOS and FAO. These metabolic adaptations are thought to accommodate various functional outputs. For instance, intracellular metabolites such as succinate and itaconate serve not only as TCA cycle intermediates, but are also closely linked to cytokine production, macrophage polarization and microbial killing.

As such, the shift from OXPHOS to glycolysis upon activation, known as the Warburg effect, has been adopted as a core paradigm in the field of immunology. Currently, the field is dominated by studies in the murine system which primarily investigate the influence of TLR ligands such as LPS on cellular metabolism. Few studies have investigated this switch in human immune cells and it is unclear whether all microbial stimuli induce the Warburg effect. To build on this, we examined whether different microbial stimuli could induce the Warburg effect in human monocytes. Whole pathogen lysates were used to represent both gram-positive and gram-negative bacteria, whereas TLR4 (LPS) and TLR2 (Pam\textsubscript{3}CysSK\textsubscript{4} (P3C)) ligands were used at different concentrations for mechanistic studies. Additionally, we investigated the consequences of metabolic rewiring on two functional outputs: cytokine production and phagocytosis. To our surprise we discovered that a shift from OXPHOS to glycolysis was only observed in monocytes stimulated with concentrations of 1-100 ng/mL LPS, but not in monocytes stimulated with P3C or other bacterial lysates. In monocytes stimulated with P3C increased OXPHOS was needed for retaining their phagocytic capacity and cytokine production. These findings therefore challenge the notion that a shift from OXPHOS to glycolysis underlies activation of all immune cells upon microbial stimulation. Instead, we propose that each individual stimulus induces a complex set of metabolic programs which govern the function of a given immune cell.
METHODS

Healthy Volunteers
Peripheral blood mononuclear cells (PBMCs) were isolated from blood donated by healthy male volunteers after informed consent. Ethical approval was obtained from the CMO Arnhem-Nijmegen.

Isolation of PBMCs and CD14+ Monocytes
Isolation of PBMCs was performed by differential centrifugation over Ficoll-Paque™ PLUS (GE Healthcare Biosciences). Monocytes were separated from the PBMCs by hyper-osmotic density gradient centrifugation over Percoll (Sigma-Aldrich), and afterwards washed once with PBS. Cells were counted in a Coulter counter (Coulter Electronics). Alternatively, CD14+ monocytes were purified from freshly isolated PBMCs using MACS microbeads for positive selection, according to the manufacturer’s instructions (Miltenyi Biotec, Germany).

Stimulation Experiments
2 x 10^5 monocytes per well were used for Seahorse measurements, while 1 x 10^6 monocytes per well monocytes were used for cytokine, RNA, and Western blot analysis. Monocytes were cultured in RPMI 1640 (no glucose, Gibco) supplemented with 10 μg/mL gentamicin (Gibco), 10 mM pyruvate (Gibco), 10 mM HEPES (Sigma-Aldrich), 5.5 mM glucose (Sigma-Aldrich) and 10% human pool serum and stimulated with either RPMI, 0.1 to 10 μg/mL Pam3CysSK4 (EMC Microcollections, Germany), 0.1 to 100 ng/mL lipopolysaccharide (LPS) from Escherichia coli (Sigma-Aldrich), 10 μg/mL poly (I:C) (Invivogen), 5 μg/mL Mycobacterium tuberculosis (H37Rv) lysate, 1 x 10^6 organisms/mL Escherichia coli (ATCC 35218) or 1 x 10^6 organisms/mL Staphylococcus aureus (clinical isolate). Unless stated otherwise, monocytes were stimulated with 10 ng/mL LPS or 10 μg/mL P3C for 24 h.

Monocytes were treated with 2-Deoxyglucose (2DG; Sigma-Aldrich) to block glycolysis, with rotenone (Sigma-Aldrich) in combination with antimycin A to block complex I of the electron transport chain, with TRIF inhibitory peptide (Pepinh-TRIF, InvivoGen) or control peptide (Pepinh-Control, InvivoGen) to inhibit TRIF-signaling, with GW9962 (Sigma-Aldrich) to inhibit PPARγ, with rosiglitazone (Sigma-Aldrich) to activate PPARγ or dimethyl itaconate (Sigma Aldrich). For experiments related to hypoxia, monocytes were stimulated with LPS or P3C for 24 h and then incubated in hypoxic (1 % oxygen) or normoxic (20 % oxygen) conditions for 2 h prior to measuring the phagocytic capacity of the monocytes.
Cell culture supernatants were collected and stored at −20°C. Cells were lysed in TRizol reagent (Invitrogen) and stored at −80°C until RNA isolation was performed or stored at −80°C until processed for Western blot analysis as described below.

**Cytokine Measurements**

The production of interleukin (IL)-1β and tumour necrosis factor (TNF)-α were measured by commercial ELISA kits (R&D Systems, Minneapolis, MN), IL-6 and IL-10 concentrations were measured by ELISA (Sanquin, Amsterdam, Netherlands).

**Lactate Measurements**

Lactate was measured from cell culture supernatants after perchloric acid precipitation. The resulting supernatants were neutralised with NaOH. An enzymatic assay in which lactate was oxidized and the resulting H$_2$O$_2$ was coupled to the conversion of Amplex® Red reagent to fluorescent resorufin by HRP (horseradish peroxidase) was used to determine lactate levels. Either 30 μL of lactate standard or 200-fold diluted sample was added to a black 96-well flat-bottom plate followed by 30 μL of reaction mix which consisted of 0.6 μL of 10 U/mL HRP (Sigma-Aldrich), 0.6 μL of 100 U/mL lactate oxidase (Sigma), 0.3 μL of 10 mM Amplex® Red reagent (Life Technologies), and 28.5 μL PBS. The assay was incubated for 20 min at room temperature and the fluorescence of resorufin (excitation/emission maxima=570/585 nm) was measured on a 96-well plate reader (Biotek).

**Extracellular flux analysis**

Real-time oxygen consumption rates (OCR) and extracellular acidification rates (ECAR) of monocytes were analyzed using an XF-96 Extracellular Flux Analyzer (Seahorse Bioscience). In short, basal metabolic rates of monocytes seeded in quintuplicate were determined during four consecutive measurements in unbuffered Seahorse medium (8.3 g DMEM powder, 0.016 g phenol red and 1.85 g NaCl in 1 L milli-Q, pH set at 7.4 at 37 °C; sterile-filtered) containing 5.5 mM glucose and 2 mM L-glutamine. After three basal measurements, three consecutive measurements were taken upon the addition of each 1.5 μM oligomycin, 1 mM FCCP, and 2 μM antimycin together with 1 μM rotenone. Glucose (20 mM) and pyruvate (1 mM) were added together with FCCP to fuel maximal respiration. All compounds used during the Seahorse runs were acquired from Sigma. Spare Respiratory Capacity (SRC) was determined as absolute increase of OCR after FCCP injection compared to basal OCR. Equal cell numbers in the wells were assessed by measuring total DNA content using the Quant-it™ dsDNA Assay Kit (Thermofisher Scientific). 96-well plates for Seahorse measurements were pre-treated with Corning™ Cell-Tak Cell and Tissue Adhesive.
Mitochondrial respiratory chain enzyme measurements

Assays for the measurements of the activity of the mitochondrial respiratory chain enzymes and citrate synthase were based on previously described methods. The assays were performed on a KoneLab autoanalyzer as described before.

Western Blotting

Monocytes were lysed in 100 μL lysis buffer (1M Tris pH 7.4, 0.5M EDTA, 5M NaCl, 10% ND40, 0.5M NaF, 25% sodium deoxycholate, PhosSTOP (Roche) and cOmplete (Roche)). The cell homogenate was frozen, thawed and processed for Western blot analysis according to the manufacturer’s instructions. Western blotting was carried out using Mini-PROTEAN TGX precast Gels (Bio-Rad). Proteins were transferred using the Trans-Blot® Turbo™ system (Bio-Rad) according to the manufacturer’s instructions. The following primary antibodies were used: actin (Sigma), phospho-AKT (S473) (Cell Signalling), total AKT (Cell Signalling), hydroxylated HIF-1α (Cell Signalling), PARP (Cell Signalling) and Caspase-3 (Cell Signalling). Unmodified, full scans of the blots can be found in Supplementary Figure 7 and 8.

Phagocytosis assay

For the phagocytosis assay, 150,000 CD14+ monocytes were seeded into a 96-well plate and stimulated for 24 h with RPMI, LPS (10 ng/mL) or P3C (10 μg/mL) (4 replicates each). Inhibitors (1 μM rotenone, Sigma-Aldrich; 1 mM 2DG, Sigma-Aldrich) were added to the cells 30 min prior to the phagocytosis assay. Phagocytosis was assessed using the Vybrant® Phagocytosis Assay Kit (Molecular Probes).

Cell Viability Assessment by Annexin-V Staining

The percentage of cells that underwent early or late apoptosis was determined by labelling with annexin V–fluorescein isothiocyanate (FITC, Biovision) and staining with propidium iodide (PI, Sigma Aldrich), according to the manufacturer’s instructions. Treated CD14+ were gently scraped after incubation with versene solution (Gibco) for 15 min on ice, washed with PBS and re-suspended in 200 μL of RPMI. Cells were incubated on ice in the dark with 1 μL of Annexin V–FITC for 15 min followed by a 5 min incubation with 1.5 μL of PI. The relative level of apoptotic cells was detected by flow cytometry within 1 h, using a FC500 flow cytometer (Beckman Coulter) and data were analysed using Kaluza 1.3 software (Beckman Coulter).

RNA Isolation and qPCRs

RNA was isolated from CD14+ cells using TRIzol reagent (Invitrogen), according to the manufacturer’s protocol. RNA was transcribed into complementary DNA by reverse-transcription using the iScript cDNA synthesis kit (BIORAD). Primer sequences used for Quantitative real-time PCR (qPCR) are listed in supplementary Table 1. Power SYBR Green
PCR Master Mix (Applied Biosystems) was used for qPCR in an AB Step one plus real-time PCR system (Applied Biosystems, Life technologies) or the CFX384 Real-Time PCR Detection System (Biorad). qPCR data were normalized to the housekeeping gene human β2M.

**Metabolite Measurements**

Three million CD14\(^+\) monocytes were seeded in each well of a 6-well plate and stimulated for 24 h with RPMI, LPS (10 ng/mL) or P3C (10 μg/mL) (2 replicates each). Dry cell pellets were flash frozen and stored at -80°C. Of note, the same samples were used for both transcriptomics and metabolomics. Samples were shipped to Metabolon, Inc for processing and measurement. 512 compounds of known structural identity were detected. Samples were prepared using the automated MicroLab STAR\(^\text{®}\) system from Hamilton Company. Raw data was extracted, peak-identified and QC processed using Metabolon’s hardware and software. Peaks were quantified using area-under-the-curve. Metabolite data normalized to protein concentration (measured by Bradford assay by Metabolon Inc.) was used for all analyses. MetaboAnalyst 3.0 was used to explore and characterise the dataset. Data was auto-scaled and an unsupervised analysis using principal component analysis (PCA) was performed to determine clustering and separation.\(^{36}\) A heatmap representing the top 25 metabolites ranked by ANOVA was generated.

**Microarray analysis**

RNA was purified from human CD14\(^+\) monocytes using TRIzol reagent (Invitrogen) followed by an additional round of purification with RNeasy Minikit columns (Qiagen, Venlo, the Netherlands). RNA quality was assessed using RNA 6000 nanochips on the Agilent 2100 bioanalyzer (Agilent Technologies, Amsterdam, the Netherlands). Purified RNA (100 ng) was labelled with the Affymetrix WT PLUS reagent kit (Affymetrix, Santa Clara, CA, USA) and hybridized to an Affymetrix Human Gene 1.1 ST array plate (Affymetrix, Santa Clara, CA, USA). Hybridization, washing and scanning were carried out on an Affymetrix GeneTitan platform according to the manufacturer’s instructions. Arrays were normalized using the robust multi array average method.\(^{37,38}\) Probe sets were defined according to Dai et al.\(^{39}\) In this method probes are assigned to Entrez IDs as a unique gene identifier. The p-values were calculated using an intensity-based moderated t-statistic (IBMT).\(^{40}\) The microarray data have been submitted to the Gene Expression Omnibus (accession number GSE78699). Expression changes in whole blood ex vivo (GSE55375)\(^{41}\) and in vivo (GSE6269)\(^{42}\) were extracted from publically available microarray data sets using the analysis pipeline described above.
Electron Microscopy

For EM analysis of mitochondria, cells were fixed in 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) and were postfixed for 1 h in 1% osmium tetroxide and 1% potassium ferrocyanide in 0.1 M cacodylate buffer. After being washed in buffer, cells were dehydrated in an ascending series of aqueous ethanol and were subsequently transferred via a mixture of propylene oxide and Epon to pure Epon 812 as embedding medium. Ultrathin gray sections (60–80 nm) were cut, contrasted with aqueous 2% uranyl acetate, rinsed, and counterstained with lead citrate, air dried, and examined in a JEOL JEM1010 electron microscope (JEOL, Welwyn Garden City, UK) operating at 80 kV.

Statistics

For normally distributed parametric data the student’s t-test was used. For non-parametric data the Wilcoxon signed-rank test was used.

Data availability

The data that support the findings of this study are available from the corresponding author upon request. Microarray data is publically available through GEO using accession number GSE78699.
RESULTS

LPS uniquely downregulates OXPHOS compared to other bacterial stimuli

CD14+ human monocytes were stimulated with a panel of commonly used concentrations of TLR ligands (LPS or P3C) or whole pathogen lysates (E. coli, S. aureus or M. tuberculosis) for 24 h to investigate changes in metabolism and cytokine production. Increased extracellular acidification rate (ECAR) (Figure 1a) and lactate production (Figure 1b) in response to all stimuli suggested a universal increase in glycolysis upon microbial stimulation. This was accompanied by changes in gene expression of enzymes in the glycolysis pathway. Specifically, expression of the first enzyme of glycolysis, hexokinase 2 (HK2), and the rate limiting enzyme, fructose 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 (PFKFB3), was increased by all stimuli (Figure 1c). Cytokine production (TNFα, IL-6, IL-1β, and IL10), a function that has been linked to changes in cellular metabolism, was also increased by all stimuli (Figure 1d).

Next, we assessed the oxidative capacity of monocytes by measuring oxygen consumption rate (OCR) and spare respiratory capacity (SRC). SRC is an indicator of the total mitochondrial capacity of a cell, which represents the amount of spare energy available during periods of stress. As reported earlier, LPS lowered basal OCR and SRC of human monocytes. In contrast P3C and bacteria lysates increased basal OCR and SRC (Figure 2a, b). Supporting these results, LPS decreased the activity of several enzymes in the mitochondrial electron transport chain (ETC), whereas P3C increased their activity (Figure 2c). Similarly, microarray analysis revealed lower expression levels of genes that comprise the five ETC complexes in monocytes stimulated with LPS compared to P3C (Figure 2d).

Protein levels of classical regulators of energy metabolism, AKT and HIF1α, were equally activated after LPS or P3C stimulation (Figure 2e), suggesting that these regulators are unlikely to control OXPHOS. Mitochondrial function has recently been suggested to be linked to mitochondrial morphology. Using electron microscopy, the mitochondria appeared to be larger in LPS- compared to P3C-stimulated monocytes (Figure 2f).
Figure 1 | Glycolysis is upregulated in human monocytes after stimulation with various pathogenic stimuli for 24 h. a–d, ECAR (a), lactate production (b), expression of glycolytic enzymes (c) and cytokine production (d) of human monocytes treated with TLR ligands or whole-pathogen lysates were measured. All data (means ± s.e.m.) are from two experiments with a total of six to eight donors. *P < 0.05, **P < 0.01, ***P < 0.001 (paired two-tailed Student’s t-test).
OXPHOS in human monocytes

Figure 2 | LPS is unique in downregulating OXPHOS in human monocytes. a, Basal OCR and real-time changes in the OCR of human monocytes stimulated with TLR ligands or whole-pathogen lysates, assessed during sequential treatment with oligomycin, carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) and antimycin A + rotenone. b, Basal OCR and SRC of monocytes treated with TLR ligands or whole-pathogen lysates. c, Enzyme activity of mitochondrial complexes (C) I–IV (Q, ubiquinone) and citrate synthase (CS) in monocytes stimulated with LPS or P3C, normalized to untreated monocytes. d, Gene expression data of mitochondrial complexes I–V of monocytes stimulated with LPS or P3C. Expression levels are presented as signal log ratios (SLR), with red representing higher and blue lower gene expression in LPS- versus P3C-stimulated monocytes. e, Immunoblot of phosphorylated AKT (pAKT), total AKT and hydroxylated HIF-1α in human monocytes stimulated with LPS or P3C for 30 or 120 min. R denotes untreated cells. HSP90 served as a loading control. f, Mitochondrial morphology analysed by electron microscopy. Data (means ± s.e.m.) are from two experiments with a total of six to eight donors (a,b), two experiments with a total of seven donors (c), one experiment with five donors (d), a representative experiment with three donors (e) or one experiment with one donor (f). *P < 0.05, **P < 0.01 (paired two-tailed Student’s t-test).
The transcriptome of LPS- vs. P3C-treated monocytes reveals specific patterns of metabolic rewiring

To validate the expression profiles of LPS- and P3C-stimulated monocytes we used a publicly available data set of \textit{ex vivo} LPS- or P3C-stimulated whole blood. Principle component analysis (PCA) using genes of selected KEGG-derived metabolic pathways revealed a clear separation of untreated vs. LPS- vs. P3C-treated cells in both data sets (Figure 3a, b). We then compared the expression levels of individual genes in each metabolic pathway and as expected observed robust changes in gene expression induced by LPS- or P3C-stimulated monocytes (Supplementary Figure 1a). Overall, LPS induced a decrease in expression of metabolic genes compared to P3C, most noticeably those part of the TCA cycle, OXPHOS and PPAR signalling pathways (Figure 3c, Supplementary Figure 1a). These observations were validated in the \textit{ex vivo} whole blood data set (Figure 3d).

Of note, a similar PCA analysis performed with publically available expression data of PBMCs from individuals with acute \textit{E. coli} vs. \textit{S. aureus} infections showed clustering that was not as distinct as LPS- vs. P3C-stimulated monocytes (Supplementary Figure 1b). Similarly, the metabolic gene expression profiles of LPS- vs. P3C-stimulated monocytes showed little overlap with the expression profiles of PBMCs from individuals with acute \textit{E. coli} vs. \textit{S. aureus} infection (Supplemental Figure 1c). Unlike a pure population of monocytes, PBMCs are a heterogeneous mix of circulating immune cells with distinct bio-energetic profiles and distribution of respiratory chain proteins \textsuperscript{15}.

LPS-treated monocytes displayed a pronounced decrease in the expression of genes involved in lipid handling and usage (Fig. 3d and Supplementary Fig. 1a). Many genes in this pathway, including PPAR-γ and its targets (LPL, FABP4 and PDK4), had lower expression in LPS- versus P3C-stimulated monocytes, suggesting that PPAR-γ may regulate the difference in OXPHOS between the two stimuli (Fig. 3e). To test this hypothesis, we inhibited PPAR-γ using the antagonist GW9662 and found that basal OCR was mostly unaffected (Supplementary Fig. 2a). GW9662 decreased IL-1β and increased IL-10 levels (Supplementary Fig. 2b). Treatment with rosiglitazone, a PPAR-γ agonist, had little effect on OCR and cytokine production in LPS- and P3C-stimulated monocytes (Supplementary Fig. 2a,b). Together, these results do not implicate PPAR-γ as the primary regulator of the oxidative capacity of P3C-stimulated monocytes. Instead, PPAR-γ may be upregulated as a consequence of the higher levels of polyunsaturated fatty acids found in monocytes stimulated with P3C compared with LPS (Fig. 3f). These fatty acids would function as natural ligands of PPAR-γ.
The TIR-domain-containing adapter-inducing interferon-β (TRIF) pathway is specifically activated downstream of TLR4, but not TLR2. Therefore, we tested whether Poly(I:C), a TLR3 ligand which signals exclusively via TRIF, would induce a similar pattern of metabolic rewiring to LPS. As with P3C, stimulation with Poly(I:C) increased ECAR and OCR (Supplementary Figure 2c), suggesting that TRIF is not involved in the metabolic rewiring of LPS-treated cells. Additionally, inhibition of TRIF using an inhibitory peptide did not affect cytokine secretion or metabolic gene expression of LPS-treated cells (Supplementary Figure 2d,e).

LPS has dose-dependent effects on OXPHOS in human monocytes

The concentrations of 10 ng/mL LPS and 10 µg/mL P3C used in our experiments are most commonly applied throughout literature. To test if the differences in regulation of OXPHOS were dose-dependent, we stimulated monocytes with a range of LPS (from 0.1 ng/mL to 100 ng/mL) or P3C (from 0.1 µg/mL to 10 µg/mL) doses and measured metabolic and functional outcomes. Various doses of LPS (1-100 ng/mL) induced greater levels of IL-1β (Figure 4a) and other cytokines (Supplementary Figure 3a) compared to P3C, whereas the lowest dose of LPS (0.1 ng/mL) was similar to P3C. Basal ECAR was similar for all conditions, however OCR and SRC varied across the different doses of LPS but not P3C (Figure 4c). The higher doses of LPS (1-100 ng/mL) decreased the oxidative capacity of monocytes whereas the lowest dose (0.1 ng/mL) increased OXPHOS similarly to P3C.

To test if these changes were reflected at a transcriptional level, we measured the expression of genes involved in glycolysis (PFKFB3 and GAPDH) and fatty acid metabolism (PPARγ, LPL, LIPA, and CPT-1α) (Figure 4d, e). In line with our findings, expression of these genes in monocytes stimulated with the low dose of LPS (0.1 ng/mL) was comparable with monocytes stimulated with P3C (10 µg/mL), but not 10 ng/mL LPS.

Lastly, time-dependent differences in OXPHOS were observed between the standard doses of 10 ng/mL LPS and 10 µg/mL P3C. 4 h post-treatment with LPS the basal OCR and SRC rates of stimulated monocytes were increased (Figure 4f). With the exception of E. coli, the other stimuli had no effect on OCR and SRC 4 h post-stimulation (Figure 4f). Glycolysis, as measured by ECAR and lactate production, was similar between all stimuli (Supplementary Figure 3b, c).
Figure 3 | Human monocytes stimulated with LPS versus P3C show differential expression of metabolic genes. a–d, Microarray analysis of human monocytes stimulated with LPS (10 ng/ml) or P3C (10 μg/ml) for 24 h (monocytes), and whole blood stimulated with LPS (1 ng/ml) or P3C (200 ng/ml) for 24 h (whole blood; data retrieved from Gene Expression Omnibus accession no. GSE55375). Shown are PCA plots based on genes from selected KEGG-derived metabolic pathways, showing the percentage of explained variance (expl. var.) (a,b); heat map of genes from selected KEGG-derived metabolic pathways in human monocytes. Expression data are presented as the SLR of LPS versus P3C. For each pathway, significantly different upregulated (red) or downregulated (blue) genes (q < 0.01) are shown (c); and heat map of genes from selected KEGG-derived metabolic pathways in human monocytes and whole blood. Expression data are presented as SLR of LPS versus P3C. For each pathway, the top 15 most differentially higher (red) or lower (blue) expressed genes in LPS- versus P3C-stimulated monocytes are shown. Significantly different regulated genes (q < 0.01) are shown in bold (d). e, Relative gene expression of LPS- and P3C-stimulated monocytes measured by real-time quantitative PCR. Heat map showing the relative abundance of polyunsaturated fatty acids in LPS- versus P3C-stimulated monocytes per donor (A–E). Data (means ± s.e.m.) are from one experiment with five donors (a,c,d,f) or from two experiments with a total of eight donors (e). *P < 0.05, **P < 0.01; (paired two-tailed Student’s t-test).
Differences in expression of genes involved in glycolysis and fatty acid metabolism were less pronounced after 4 h compared to 24 h between the two conditions. (Figure 4g, h). These transcriptional differences in kinetics were validated in the whole blood data set stimulated with LPS vs. P3C (Supplementary Figure 3 d,e).

Although metabolic rewiring can be dependent on the maturation and activation state of cells, measurement of HLA-DR revealed no differential effect on activation by either 0.1 or 10 ng/mL LPS or 10 µg/mL P3C (Supplementary Figure 4a,b). P3C-stimulated monocytes expressed slightly more CD11c and CD83 compared to LPS. Nevertheless, stimulation with 0.1 ng/mL LPS and 10 ng/mL LPS led to a similar maturation pattern despite differences in OXPHOS, suggesting that the extent of monocyte maturation probably cannot explain differences in metabolic rewiring upon LPS- and P3C-stimulation.

**Functional consequences: OXPHOS is needed for phagocytosis, whilst cytokine production relies on both OXPHOS and glycolysis in P3C-stimulated monocytes**

We next investigated how cytokine production and phagocytosis are influenced by the different metabolic programs induced by LPS or P3C. As described elsewhere, inhibition of glycolysis with 2DG decreased IL-1β (Figure 4a) and other cytokines (Supplementary Figure 5a) in both conditions. Inhibition of OXPHOS using the complex I inhibitor rotenone, lowered cytokine production only in P3C-stimulated cells (Figure 4b). This points to a P3C-specific reliance on OXPHOS for cytokine production. In contrast to Kelly et al., IL-10 levels were decreased in rotenone-treated cells after LPS-stimulation (Supplementary Figure 5b.) Rotenone did not affect cell death, as assessed by annexinV/PI-staining and measurement of cleaved caspase-3 and PARP (Supplementary Figure 5c, d).

Next we determined the phagocytic capacity of stimulated monocytes and found that LPS compared to P3C lowered phagocytic rates (Figure 5c). To determine whether OXPHOS facilitated the higher phagocytic capacity of P3C- vs. LPS-treated cells, we inhibited complex I of the ETC with rotenone. Treatment with rotenone significantly decreased the phagocytic capacity of P3C-stimulated monocytes whereas untreated or LPS-treated monocytes were unaffected. Nonetheless, lowering of oxygen consumption using hypoxic culture conditions (1% vs. 5% of O₂), had no effects (Supplementary Figure 5e). 2DG also did not affect the phagocytic capacity of stimulated monocytes, suggesting that OXPHOS but not glycolysis is an important determinant of the phagocytic capacity of human monocytes (Figure 4c).
Figure 4 | Dose- and time-dependent metabolic rewiring in human monocytes. a–e, IL-1β production (a), basal ECAR (b), basal OCR and SRC (c), and relative gene expression (d,e) of human monocytes treated with various doses of LPS and P3C for 24 h. f–h, Basal OCR and SRC (f) and relative gene expression (g,h) of human monocytes treated with TLR ligands or whole-pathogen lysates for 4 h. Data (means ± s.e.m.) are from one experiment with five donors (a–e), from two experiments with a total of five to six donors (f) or from two experiments with a total of eight donors (g,h). *P < 0.05, **P < 0.01; exact p values are also indicated in the graphs (paired two-tailed Student's t-test).
Metabolite analysis reveals pronounced differences in TCA cycle metabolites between LPS- and P3C-stimulated monocytes

To potentiate our understanding of differences in metabolic rewiring between LPS- and P3C-treated monocytes, we measured intracellular levels of over 500 metabolites. PCA analysis confirmed a robust separation in metabolic responses upon LPS- and P3C-stimulation (Figure 5d). A heat map of the top 25 differentially regulated metabolites showed increased levels of succinate, citrate and itaconate specifically in LPS-treated cells, whereas gulonate levels were highly increased upon P3C stimulation (Figure 5e). These findings appear to match the two previously described breaks in the TCA cycle at citrate and succinate upon LPS stimulation\textsuperscript{18}, but indicate that these breaks might be absent in P3C-stimulated monocytes (Figure 5f). An increase in expression of IRG1, which converts citrate to itaconate, in LPS- but not P3C-stimulated cells further supports these findings (Figure 5g). Moreover, LPS stimulation decreased the expression of the two TCA cycle enzymes, IDH1 and SDH, present after each break in the TCA cycle (Figure 5g).

As shown above (Figure 5e), itaconate has been reported to accumulate intracellularly in LPS-treated mouse macrophages \textsuperscript{19}. To test if itaconate plays a functional role in cytokine production, membrane permeable dimethyl-itaconate was added prior to LPS or P3C stimulation. IL-1\textbeta, IL6 and IL-10 were all decreased, whereas levels of TNF\textalpha were mainly unaffected both upon LPS or P3C stimulation (Supplementary Figure 6a). Moreover, dimethyl-itaconate did not change glycolytic metabolism, as assessed by lactate production, in either LPS or P3C-stimulated monocytes(Supplementary Figure 6b). These results highlight the importance of intracellular metabolites in the regulation of immune cell function and demonstrate their immune-modulatory potential independent of the pathogenic stimulus.
Figure 5 | Differential metabolic rewiring of human monocytes stimulated with LPS or P3C leads to functional differences in cytokine production and phagocytosis. a, b, IL-1ß production of monocytes pretreated with 2DG (a) or rotenone (b) for 1 h before addition of LPS or P3C for 24 h. c, Phagocytosis of monocytes stimulated with LPS or P3C for 24 h and treated with 2DG or rotenone for 30 min before the start of the assay. d, PCA plot based on intracellular metabolites. e, Heatmap of the top 25 metabolites most differentially regulated in monocytes stimulated with LPS or P3C for 24 h, with red representing higher and blue representing lower abundance. f, Scheme showing the relative abundance of TCA cycle intermediates in monocytes stimulated with LPS or P3C for 24 h, with red representing higher and blue representing lower abundance. g, Relative expression of genes involved in regulating levels of TCA cycle intermediates. Data (means ± s.e.m.) are from two experiments with a total of five donors (a–c) or from one experiment with five donors (d–g). *P < 0.05, **P < 0.01 (paired two-tailed Student’s t-test).
DISCUSSION

Evidence acquired mostly from in vitro stimulations of myeloid cells with LPS has pinpointed an increase in glycolysis and decrease in OXPHOS as characteristic for activated pro-inflammatory innate immune cells. Our study recapitulates these findings in human monocytes exposed to LPS, but also demonstrates that a decrease in OXPHOS upon activation of myeloid cells is not an universal response to pathogenic stimuli. Furthermore, we link different metabolic routes adopted by human monocytes upon microbial stimulation to host defence functions like cytokine release and phagocytosis.

Increased glucose utilisation upon activation with TLR-ligands or bacterial stimuli has previously been demonstrated and has led to the identification of various upstream regulators of glycolysis, including AKT and HIF1α. Our studies confirm these findings in human monocytes, as inhibition of glycolysis with 2DG reduced IL-1β production after stimulation with LPS or P3C. Although some conflicting results in murine dendritic cells versus macrophages have been reported, in human monocytes 2DG also decreased TNF-α, IL-6 and IL-10 production. Together with higher glycolytic rates, LPS-stimulated monocytes displayed decreased OXPHOS and metabolic flexibility. In contrast, stimulation with P3C or lysates from E. coli, S. aureus or M. tuberculosis resulted in increased or unaltered OXPHOS and metabolic flexibility. Thus, the classical Warburg effect induced by LPS is not a universal prerequisite for pro-inflammatory cytokine responses.

Pathogen-specific metabolic rewiring may fuel different functional outputs. In this study we show that the phagocytic capacity of human monocytes is related to their oxidative capacity. LPS-stimulation lowered OXPHOS as well as the phagocytic capacity of monocytes in comparison to P3C-stimulation. Similarly, inhibition of complex I of the ETC with rotenone decreased the phagocytic capacity of P3C-stimulated monocytes. Phagocytosis is an energy demanding process and OXPHOS unlike glycolysis is characterised by a high ATP yield. Thus, OXPHOS seems to be more capable of supporting the bio-energetic demands of phagocytosis. Interestingly, the phagocytic capacity of monocytes was unaffected by short-term hypoxia which, whilst activating HIF1α, may not result in complete inhibition of mitochondrial respiration.

Previous studies have shown that macrophages are dependent on several metabolic pathways for phagocytosis, making it likely that other pathways such as FAO contribute to phagocytosis as well. Similarly as seen for phagocytosis, inhibition of OXPHOS decreased cytokine production from P3C- but not LPS-stimulated monocytes, further emphasizing that different stimuli may use convergent or redundant metabolic pathways to execute their inflammatory functions.
We attempted to delineate the differences in OXPHOS between LPS- and P3C-stimulated monocytes in several ways. Firstly, we examined the contribution of TRIF signalling, since both stimuli induce MyD88-signalling, whereas only LPS has been shown to activate TRIF. Poly(I:C), a TLR3 ligand exclusively recruiting TRIF \(^{24}\), increased the oxidative capacity of monocytes, thus making it unlikely that signalling via TRIF contributes to the LPS-induced decrease in OXPHOS. Secondly, we looked at PPAR\(\gamma\), which has been shown to promote OXPHOS, stimulate mitochondrial biogenesis in mouse macrophages \(^{25}\) and has been linked to the regulation of phagocytosis in human macrophages \(^{26}\). Although expression of PPAR\(\gamma\) was greater in P3C- than LPS-stimulated cells, stimulation or inhibition of PPAR\(\gamma\) did not affect OXPHOS or the cytokine production of LPS- nor P3C-stimulated monocytes. It is more likely that the activation of PPAR\(\gamma\) by P3C is a consequence of stimulation rather than responsible for P3C-induced metabolic rewiring. Thirdly, mitochondrial morphology was visualized. A recent study linked changes in mitochondrial morphology to the metabolic differences observed between glycolytic T-effector (TE) cells and OXPHOS-dependent T-memory (TM) cells. Mitochondrial fusion in TM cells favoured OXPHOS and FAO, whereas fission in TE cells lead to loosening of the cristae which resulted in less efficient transport of electrons through the ETC and in turn promoted glycolysis as an alternative fuel source \(^{27}\). Electron microscopy revealed larger mitochondria after LPS stimulation, suggesting that changes in mitochondrial morphology might contribute to differences in OXPHOS and metabolic rewiring upon LPS- vs. P3C-stimulation. Lastly, metabolome analysis was executed and revealed increased presence of metabolites that have been shown to accumulate upon a broken TCA cycle in LPS-stimulated murine macrophages \(^{6,7}\) in LPS- but not P3C-stimulated human monocytes. These metabolites, such as citrate, succinate and itaconate, have been identified as important regulators of intracellular metabolism and inflammatory activation. For example, excess citrate is transported into the cytosol where it is converted into acetyl-CoA for fatty acid synthesis, and to oxaloacetate which promotes NO-production that can inhibit components of the mitochondrial ETC via nitrosylation \(^{28}\). These metabolites may thus serve to maintain the reduced respiratory capacity of LPS-stimulated monocytes.

Itaconate treatment of LPS-stimulated mouse macrophages has been shown to dampen the inflammatory response by modulating mitochondrial respiration \(^{29}\). Likewise, pretreatment of monocytes with itaconate lowered cytokine release not only in LPS- but also in P3C-stimulated cells, demonstrating that itaconate can modulate the inflammatory response of human monocytes irrespective of the stimulus. The modulatory effects of itaconate may be relevant in septic patients, where IRG1, the enzyme responsible for itaconate production, was found to be highly up-regulated in their PBMCs \(^{30}\). Furthermore itaconate plays a key role in LPS-induced tolerance in mouse macrophages \(^{30}\).
Additionally, we have identified gulonate to be specifically induced in P3C-stimulated monocytes. Gulonate is part of the glucuronic acid pathway, an alternative pathway for the oxidation of glucose that produces activated UDP-glucuronate. Gulonate is ultimately converted to D-xylulose-5-phosphate that enters the pentose phosphate pathway. Therefore, gulonate and gulonate-derived metabolites may represent an alternative pathway to oxidize glucose in P3C-treated cells. Specific metabolites that accumulate in P3C-treated cells, including gulonate, may preserve OXPHOS through as yet unidentified mechanisms.

Although LPS and P3C are often used as a model for gram-negative bacteria (like *E. coli*) and gram-positive bacteria (like *S. aureus*) respectively, *ex vivo* stimulations of human monocytes with either LPS, *E. coli*, P3C or *S. aureus* led to varied metabolic profiles. In the case of LPS, a low dose (0.1 ng/mL) induced similar metabolic changes to P3C, whereas higher doses led to the characteristic decrease in OXPHOS. This suggests that differences in signalling strength rather than qualitative signalling differences between LPS and P3C explain the divergent metabolic phenotype. Of much interest however are the parallels of low dose LPS (0.1 ng/mL) with acute *in vivo* infections of *E. coli* and of high dose LPS (1-100 ng/mL) with sepsis which has recently been characterised by defects in OXPHOS and energy metabolism in leukocytes. Concentrations above 0.1 ng/mL of LPS seem to cross a certain signalling threshold at which not only is glycolysis increased, but OXPHOS is also reduced to maintain ATP production. These dose-related effects therefore emphasize the complexity of intracellular metabolic adaptations and the importance of selecting representative *in vitro* models for disease.

This study highlights the complexity of metabolic rewiring in activated human immune cells and demonstrates the importance of understanding pathogen-specific metabolic reprogramming. We propose that each pathogen induces a highly specific metabolic program in human innate immune cells, thereby determining the functional output of the cells. Whilst some functional outputs, e.g. cytokine release, may be relatively similar, the underlying metabolic routes may vary significantly. Targeting metabolic routes to alleviate inflammatory conditions or improve antimicrobial host defence thus holds therapeutic promise, yet tailor-made approaches are warranted.
AUTHOR CONTRIBUTIONS

EL, LB, JMR, AH and RS conducted the majority of the experiments and data analysis. GH performed all data analysis related to the transcriptome and metabolome results. RR and RK assisted in the experiments related to assessing mitochondrial function. JF performed the electron microscopy. LJ, RH, RC, MGN critically contributed to the design of the study. EL, LB, JMR, MGN and RS wrote the manuscript together.

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18. O'Neill LA, Kishton RJ, Rathmell J. A guide to


Supplementary Figure 1 | Microarray analysis of the following datasets: human monocytes stimulated with LPS (10 ng/mL) or P3C (10 μg/mL) for 24 h (ex vivo; monocytes), or PBMCs isolated from patients with an E. coli or S. aureus infection (in vivo; PBMCs; data retrieved from GSE6269). (a) Heat map of genes from selected KEGG-derived metabolic pathways in the ex vivo monocyte dataset. Expression data are presented as signal log ratio (SLR) of LPS vs. P3C, LPS vs. RPMI or P3C vs. RPMI. For each pathway significantly different regulated genes (q<0.01) are shown. Genes with a SLR>1.5 or SLR<-1.5 are highlighted in bold. (b) PCA plot of the in vivo PBMC dataset based on genes part of selected KEGG-derived metabolic pathways. (c) Heat map of genes of selected KEGG-derived metabolic pathways. Expression data are presented as signal log ratio (SLR) of LPS vs. P3C or E. coli vs. S. aureus. For each pathway the top fifteen most differentially higher (red) and lower (blue) expressed genes in LPS- vs. P3C- stimulated monocytes are shown.
Supplementary Figure 2 | (a) Basal oxygen consumption rate (OCR) of human monocytes pretreated with the PPARγ-inhibitor GW9662 for 1 h before addition of LPS or P3C for 24 h. (b) Cytokine production of human monocytes pretreated with GW9662 or the PPARγ-activator rosiglitazone for 1 h before addition of LPS or P3C for 24 h. (c) Basal extracellular acidification rate (ECAR), OCR and spare respiratory capacity (SRC) of human monocytes treated with various TLR ligands for 24 h. (d, e) Cytokine production (d) and gene expression (e) of human monocytes pretreated with either a TRIF-specific inhibitory peptide or a control peptide for 1 h before addition of LPS or P3C for 24 h. Data (mean ± s.e.m.) are from one experiment with five donors (a), one experiment with three to five donors (b), two experiments with a total of eight donors (c) or one experiment with three donors (d, e). * p<0.05, ** p<0.01 (paired two-tailed t-test).
Supplementary Figure 3 | (a) Cytokine production of human monocytes treated with several doses of LPS and P3C for 24 h. (b) Basal extracellular acidification rate (ECAR) and lactate production of human monocytes stimulated with TLR ligands or whole pathogen lysates for 4 h. (c) Basal oxygen consumption rate (OCR) and real-time changes in OCR of human monocytes stimulated with TLR ligands or whole pathogen lysates for 4 h, assessed during the sequential treatment with oligomycin, FCCP and antimycin A/rotenone. (d, e) Microarray analysis of the following datasets: human monocytes stimulated with LPS (10 ng/mL) or P3C (10 μg/mL) for 24 h (ex vivo: monocytes), and whole blood stimulated with LPS (1 ng/mL) or P3C (200 ng/mL) for 1-24 h (ex vivo: whole blood; data retrieved from GSE55375). (d) PCA plot of the whole blood dataset based on genes part of selected KEGG-derived metabolic pathways. (e) Heat map of genes part of selected KEGG-derived metabolic pathways. Expression data are presented as signal log ratio (SLR) of LPS vs. P3C. For each pathway the top fifteen most differentially increased (red) or decreased (blue) genes in LPS- vs. P3C-stimulated monocytes are shown. Data (mean ± s.e.m.) are from one experiment with five donors (a) or from two experiments with a total of five to six donors (b-c). * p<0.05, ** p<0.01 (paired two-tailed t-test).
Supplementary Figure 4 | Maturation markers present on human monocytes stimulated with LPS or P3C for 24 h. (a) Representative flow cytometry plots from one donor. (b) Mean fluorescent intensity (MFI) representing the presence of maturation markers in monocytes of three donors. Data (mean ± s.e.m.) are from one experiment with three donors.
Supplementary Figure 5 | (a, b) Cytokine production of monocytes pretreated with either 2-Deoxyglucose (2DG) (a) or rotenone (b) for 1 h before addition of LPS or P3C for 24 h. (c, d) Cell death in monocytes pretreated with rotenone for 1 h prior to addition of LPS or P3C for 24 h, assessed by annexinV-PI-staining (c) and Western blot for PARP and cleaved caspase-3 (d). (e) Phagocytic capacity of monocytes stimulated with LPS or P3C for 24 h and held under normoxic or hypoxic conditions for 2 h prior to the start of the assay. Data (mean ± s.e.m.) are from two experiments with a total of five donors (a-b), or one experiment with three donors (c-e). * p<0.05, ** p<0.01 (paired two-tailed t-test).
Supplementary Figure 6 | Cytokine (a) and lactate (b) production of human monocytes pretreated with dimethyl itaconate (DI) for 1 h before addition of LPS or P3C for 24 h. Data (mean ± s.e.m.) are from one experiment with three donors. * p<0.05, ** p<0.01 (paired two-tailed t-test).
Supplementary Figure 7 | Original scans and figures of Western blot images used in Figure 2e. The box indicates the relevant samples.
Supplementary Figure 8 | Original scans and figures of Western blot images used in Supplementary Figure 5d. The box indicates the relevant samples.
### Supplementary Table 1  
Primer sequences used to examine gene expression levels by RT-qPCR.

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Chapter 7

Tissue metabolic changes drive cytokine responses to *Mycobacterium tuberculosis*
Chapter 7

ABSTRACT

Cellular metabolism can influence host immune responses to Mycobacterium tuberculosis (Mtb). Using a systems approach, differential expression of 292 metabolic genes involved in glycolysis, glutathione, pyrimidine and inositol phosphate pathways was evident at the site of a human tuberculin skin test challenge in patients with active tuberculosis infection. For 28 metabolic genes, we identified single nucleotide polymorphisms (SNPs) that were trans-acting for in vitro cytokine responses to Mtb stimulation, including glutathione and pyrimidine metabolism genes that alter production of Th1 and Th17 cytokines. Our findings identify novel therapeutic targets in host metabolism that may shape protective immunity to Mtb infection.
INTRODUCTION

The activity of many cellular metabolic pathways can impact the host immune response to infections [1]. Individual metabolic pathways have been implicated in anti-mycobacterial responses: glutathione enhances IL-12 and IFN-γ secretion following Mtb stimulation [2]; tryptophan catabolism is involved in Mtb-induced production of IL-1β and IL-23 [3] and control of Mtb growth [4]; and a shift towards aerobic glycolysis in Mtb infected macrophages regulates IL-1β production [5,6]. However, as metabolic reactions are intrinsically interdependent, the challenge lies in determining the relative roles of these and other pathways during in vivo Mtb infection.

Our group has made use of the human tuberculcin skin test (TST) challenge model to faithfully reflect the inflammatory changes that occur at the site of tuberculosis (TB) disease, characterising the tissue immunological pathways induced early after mycobacterial antigen exposure [7]. However, to date, no studies have explored the metabolic changes and their functional consequences on downstream cytokine responses in such a model. Quantitative cytokine production in response to mycobacterial stimulation has been associated with genetic polymorphisms [8]. These cytokine quantitative trait loci (cQTLs) provide a functional insight into how genetics influences an inflammatory response, and in turn identify critical pathways that may be amenable to host-directed therapy [8]. In this study, we use the TST model to test the hypothesis that differential tissue expression of genes involved in regulating metabolic pathways can directly influence cytokine production following Mtb stimulation. In turn, our findings provide putative mechanistic links between the activity of cellular metabolic pathways and immune effector functions.
METHODS

Transcriptomic Data Analysis
Transcriptomic data from TST and blood of patient with active TB were derived from datasets E-MTAB-3254 and E-MTAB-3260 (https://www.ebi.ac.uk/arrayexpress/). Transcriptomes from human Mtb-infected and healthy lymph nodes (LN) were derived from dataset E-GEOD-63548. We used the KEGG pathway database (http://www.genome.jp/kegg/pathway.html) to derive 33 pathways associated with human metabolism, yielding a list of 1422 metabolic genes containing no duplicate genes and no annotation to the original pathways (Tables S1 & S2). Differential gene expression and pathway enrichment analyses, as well as Venn diagram, and network plots generation were performed as previously described [7] (Supplementary Methods for more details).

SNP extraction and cQTL mapping
Single nucleotide polymorphisms (SNPs) within metabolic genes were identified as described in Supplementary Methods. SNPs were then mapped (p<0.05 cut-off) for cQTLs in the 500FG cohort. This cohort consists of 500 healthy individuals of Dutch European ancestry from the Human Functional Genomics Project (www.humanfunctionalgenomics.org), from which peripheral blood mononuclear cells (PBMC) or macrophages have been stimulated by heat-killed Mtb and the secretion of IFN-γ, IL-17, IL-22 (7 days post-stimulation) and IL-1β, IL-6 and TNFα (24hr post-stimulation) measured, as previously described [8]. To identify cQTLs, raw cytokine levels were first log transformed then mapped to genotype data using a linear regression model with age and gender as covariates. P values were obtained using linear regression analysis of cytokine on genotype data, as previously described [8].

Metabolite reporter analysis
Reporter metabolite analysis [9] was performed in Matlab using the RAVEN Toolbox (http://biomet-toolbox.org/index.php?page=downtools-raven) and the human genome-scale metabolic reconstruction network HMR 2.00 provided in Human Protein Atlas (http://www.metabolicatlas.org/downloads/hmr) (see Supplementary Methods).
RESULTS

Metabolic gene expression at the site of TST.

We have previously demonstrated that the transcriptional response at the site of TST is characterised by upregulation of 1725 genes that closely reflect changes seen in dissected human TB granuloma relative to healthy lung tissue [8]. We extend these findings by demonstrating that the TST signature is enriched within Mtb-infected relative to healthy LN (Figure 1A), confirming that the TST transcriptional responses mirrors pathology seen at established sites of human TB disease.

Using the KEGG database for annotations of metabolic pathways, we assessed the expression of metabolic genes at the site of TST challenge in patients with active TB disease. 292 significant gene changes were observed when comparing gene expression between the site of tuberculin or saline injection (Figure S1 and Table S3). To relate these changes in tissue with those in blood we assessed metabolic gene expression in the peripheral blood transcriptome of patients with active TB compared to healthy volunteers. Changes in tissue and blood showed a remarkable lack of concordance, driven largely by the paucity (only 9) of differential expression of metabolic genes in the blood compartment (Figure 1B & Figure S2 & Table S4). This demonstrated that the TST challenge model provides greater molecular resolution to identify host differential metabolic gene expression to mycobacterial infection than the blood compartment [7].

Given the interconnected relationship between multiple metabolic pathways, we generated a network depicting the ten most enriched KEGG annotated metabolic pathways based on the 292 differentially expressed metabolic genes (Figure 1C). As expected, TST induced changes in multiple inter-linked metabolic pathways. These included not only several previously described pathways such as glycolysis and glutathione metabolism, but also others such as inositol phosphate metabolism and specific amino acid metabolic pathways not previously associated with TB [1,2]. Since differentially expressed genes within the same metabolic pathway were both up- or down-regulated (Figure S3), we evaluated which metabolites in these pathways were most affected using Reporter Metabolite analysis, identifying those metabolites in the human metabolic network around which the most transcriptional change occurs. [10]. The model predicted changes in several key metabolites between TST vs saline, including 1,3-bisphospho-D-glycerate (glycolysis), L-formylkynurenine (tryptophan metabolism), 1-phosphatidyl-1D-myo-inositol-3,4-bisphosphate and glutathione (Figure 1D). Therefore, the model predicts that gene expression changes in the TST alter the concentration of several bioactive metabolites in multiple pathways, and that this has the potential to impact the nature of the host immune response to mycobacterial antigen exposure.
Figure 1 | Metabolic pathways enriched in TST responses relative to saline injection. (A) Expression of TST signature in Mtb infected lymph nodes (LN) relative to healthy LN. Each dot represents one sample. Horizontal lines represent median value expression. * p<0.0001 by Mann-Whitney test. (B) Pairwise comparison of 292 genes differentially expressed in TST relative to saline injection relative to the expression difference between the blood of patients with active TB and healthy volunteers (HV). r = Spearman’s rank correlation coefficient, CoV = covariance. (C) The top ten most statistically enriched KEGG metabolic pathways are represented in a network plot, in which the edges indicate associations between genes (yellow nodes) and named pathways (blue nodes), and the node size is proportional to the respective pathway enrichment -log10 p value enrichment statistic. (D) Reporter metabolites with differentially expressed in TST compared to saline injection. Metabolites selected for known association with metabolic pathways and ranked by increasing p value.
Mapping metabolic gene cQTLs to Mtb-induced cytokine production

Next, we sought to test the hypothesis that metabolic changes in the tissue environment influence Mtb-induced cytokine production. We used natural genetic variation to identify putative cis-acting SNPs for metabolic genes and tested whether they might be trans-acting SNPs for cytokine responses. We identified metabolic genes differentially expressed in the TST and assessed their impact on cytokine secretion following Mtb lysate-stimulation in a cohort of 500 healthy individuals. First, we used genotypes extracted from the 1000 Genomes Project to identify 16061 SNPs from the 109 metabolic genes that comprised the 10 most enriched metabolic pathways in the TST (Figure 1C). We then assessed which of these SNPs were associated with variable cytokine secretion, generating 2376 putative cQTLs. To reduce multiple testing false positives, we focused on SNPs that were both present within the mRNA coding region of the gene of interest and influence the same gene’s transcription (i.e. metabolic gene SNPs that were cis-eQTLs).

This analysis generated 47 cQTL SNPs from 28 metabolic genes (Figures 2A-B & Table S5). Of these genes, the most over-represented metabolic pathways included the glutathione, glycolysis, inositol phosphate metabolism and pyrimidine pathways (Figure 2C). Many amino acid metabolism pathways were also observed (Figure 2C), and ALDH3A2, ALDH3A1, LDHA and IL4I1 were the most frequent constituent genes from these pathways (Figure 2C). Glutathione & pyrimidine metabolism predominantly influenced the secretion of IFN-γ & IL-17: 7 of 15 (47%) cQTLs that regulated IFN-γ secretion and 4 of 7 (57%) cQTLs that regulated IL-17 secretion were derived from genes assigned to glutathione or pyrimidine metabolic pathways, whereas no cQTLs from these pathways influenced cytokine secretion by macrophages (Figure 2B). In contrast, genes involved in glycolysis, amino acid and inositol phosphate metabolism acted as cQTLs more ubiquitously, influencing the secretion of both T cell and myeloid cell derived cytokines (IL-1β, IL-6, IL-22 and TNFα) (Figure 2B).

Finally, in order to explore the physiological relevance of the 28 metabolic genes that can act as cQTLs, we compared their gene expression in Mtb-infected LN with the transcriptional changes seen at the site of TST (Figure 2D). This revealed the expression between these tissues to be highly correlated, indicating that these metabolic genes have the potential to also exert cQTL activity at the site of human TB disease, thus shaping the local inflammatory response to Mtb infection.
Figure 2 | Identification of cQTLs within metabolic genes differentially expressed in a TST. Box plots showing the association of SNP genotypes with Mtb-induced cytokine levels. The length of the box is the interquartile range and the whiskers indicate the range of 1.5 x the length of the box from either end of the box. P values were obtained using linear regression analysis of cytokine on genotype data. (B) Heatmap of all 47 cQTL SNPs and their relationship to cytokine secretion following PBMC or macrophage (Mfs) stimulation with Mtb lysate. (C) Number of cQTL genes comprising each of the represented metabolic pathways, and number of genes representing amino acid metabolism pathways. (D) Gene expression of 28 metabolic genes with cQTL SNPs in TST relative to saline compared to the expression in Mtb infected lymph nodes (LN) relative to healthy LN. $r^2$ = Spearman's rank correlation coefficient.
DISCUSSION

Putative roles for individual metabolic pathways shaping the host response to Mtb infection have been proposed [2,3,5], but their relative contribution in a multicellular tissue infection setting has not been investigated. We took a systems approach using the human in vivo TST challenge model, revealing gene expression changes in multiple metabolic pathways that in turn predict enrichment of several bioactive metabolites. Furthermore, we show that genetic polymorphisms in these differentially expressed metabolic genes control Mtb induced cytokine production.

We have previously shown that the inflammatory response to TST challenge closely reflects the immunopathological changes in human TB disease [7], and have now extended these observations to Mtb-infected LN. Importantly, the quantitatively smaller changes in metabolic gene expression in the blood of patients with active TB illustrate the importance of studying metabolism in the tissue setting. We observed differential gene expression of multiple pathways, including glycolysis, glutathione, pyrimidine and inositol phosphate metabolism. However, to determine whether the changes in the metabolic environment after Mtb infection can directly influence the ensuing immune responses, we used the 500FG cohort to map SNPs in metabolic genes that affect cytokine secretion [8]. The expression of these genes was highly correlated between the site of TST challenge and human Mtb-infected LN, underlying that these novel metabolic gene cQTLs are likely to have functional consequences on the immune response in human TB disease.

Our analyses re-affirm previous observations that changes in glycolysis and glutathione metabolism can influence cytokine secretion following Mtb stimulation [2,5], whilst identifying new roles for pyrimidine and inositol phosphate metabolism, as well as several amino acid pathways. Pyrimidine metabolism has recently been associated with regulating inflammasome activity and cytokine secretion in the blood of older individuals [11]. We demonstrate that genes which regulate pyrimidine and glutathione metabolism predominantly impact the secretion of T cell derived cytokines IFN-γ and IL-17. In this way, our novel functional genomics approach has revealed that therapeutic manipulation of pyrimidine and glutathione pathways may influence Th1 and Th17 polarisation, and thus the balance between protection and pathology in Mtb-infected tissues [12].

In contrast, we demonstrate that differentially expressed genes involved in amino acid and inositol phosphate metabolism impact a wider array of cytokines, including those secreted from myeloid cells. Phosphatidylinositol is synthesised from glucose-6-phosphate in two steps, and is the backbone of signal transduction components such as IP3 and Akt [6]. This may explain the observed association between polymorphisms in genes of this pathway...
(INPP4A, INPP5D and PIK3C2G) and secretion of a larger range of cytokines, including IFN-γ, IL-22, IL-6 and IL-1β. Of the cQTL genes involved in amino acid metabolism, ALDH3A2 and ALDH3A1 play key roles in detoxification and lipid peroxidation [13], whereas IL4I1 expression is predominantly in the lysosomal antigen processing compartment [14], suggesting that alteration in macrophage amino acid metabolism may impact on intracellular survival of Mtb.

Our study has some limitations. Firstly, we restricted our cQTL analyses to SNPs that were also cis-eQTLs to limit multiple testing errors, thus likely missing other functionally relevant cQTLs that may act in trans via other genes. Equally, identification of SNPs that were eQTLs was limited to databases that probed blood and tissues not infected with Mtb, possibly missing other functionally relevant SNPs in the context of Mtb infection. Finally, the effector cytokines studied for cQTL analysis were restricted to the manually selected panel available in the 500FG database, introducing bias into the breadth of immunological effector functions exerted by metabolic gene SNPs.

In summary, this study made a comprehensive assessment of the human tissue metabolic transcriptional response to in vivo mycobacterial antigenic stimulation. A number of known and novel metabolic pathways were differentially expressed, and genetic variation in identified genes affected cytokine responses to Mtb. Therefore, our systems approach revealed a new layer of complexity to the host anti-mycobacterial response and provide support for host-directed strategies targeting cellular metabolism, such as the regulation of glycolysis by metformin [15]. Furthermore, our approach combining transcriptomics and functional genomics illustrates a pipeline that can be used to identify novel and clinically relevant pathways in the context of other infectious diseases.

FUNDING
This work was supported by the Wellcome Trust (Research Fellowship WT101766/Z/13/Z to GP) and by an ERC Consolidator Grant (#310372 to MGN) and a Spinoza grant of the Netherlands Organization for Scientific Research (to MGN). RvC and EL were supported by The European Union’s Seventh Framework Programme (EU FP7) project TANDEM (HEALTH-F3-2012-305279).
REFERENCES


SUPPLEMENTARY MATERIAL

Transcriptomic data analysis
Transcriptomic data from TST and blood of patient with active TB were derived from datasets E-MTAB-3254 and E-MTAB-3260 (https://www.ebi.ac.uk/arrayexpress/). This comprised 16 individuals with active TB disease who underwent both TST skin biopsy and blood transcriptome profiling. We also included 8 individuals with active TB who received saline skin injection as a comparator for the TST transcriptomic response. Additionally, the blood transcriptome of 8 healthy volunteers was analysed to assess the transcriptomic changes in the blood of the patients with active TB. Transcriptomes from human Mtb-infected (n=22) and healthy lymph nodes (LN) (n=4) were derived from dataset E-GEOD-63548.

For all datasets, probe identifiers were converted to gene symbols using platform annotations provided with each dataset. Datasets were sorted alphabetically by gene symbol, and duplicate genes were removed using Microsoft Excel duplicate remover function. Significant differences in metabolic gene expression between groups was performed by unpaired t-test with alpha p<0.01 and Bonferroni correction for multiple testing in Multi-Experiment Viewer software (http://www.tm4.org/). Pathway enrichment using InnateDB online tool was performed on the resulting gene list (http://innatedb.com/). Genes contributing to the 10 most enriched KEGG metabolic pathways were put forward for SNP and QTL analyses. Network plots were generated using Gephi v0.8.2 (https://gephi.org/), and Venn diagrams were constructed using BioVenn tool (http://www.cmbi.ru.nl/cdd/biovenn/).

SNP extraction and cQTL mapping
Single nucleotide polymorphisms (SNPs) within metabolic genes were identified from the publicly available variation database dbSNP using the NCBI Variation Viewer tool (www.ncbi.nlm.nih.gov/variation/view). SNPs located 250 kb upstream and downstream of the genes of interest with minor allele frequency ≥ 0.05 were extracted using the GRCh38.p7 assembly. Linkage disequilibrium (LD) SNP pruning using genotypes extracted from 1000 Genomes Project for Europeans (http://www.internationalgenome.org/home) based on pairwise genotypic correlation was performed using plink v1.07 (http://zzz.bwh.harvard.edu/plink/dataman.shtml) and one of a pair of SNPs was removed if the LD was greater than 0.5. LD-pruned SNPs as well as any other functional SNP (missense variants) that were removed due to high LD were then mapped (p<0.05 cut-off ) for cQTLs in the 500FG cohort. To reduce multiple testing false positives, the putative cQTLs were further refined to include only SNPs that both were present within the coding region of the gene of interest (https://genome.ucsc.edu/) and had eQTL activity for the encoded gene (http://www.gtexportal.org/home/).
Metabolite reporter analysis

Reporter metabolite analysis [9] was performed in Matlab using the RAVEN Toolbox (http://biomet-toolbox.org/index.php?page=downtools-raven) and the human genome-scale metabolic reconstruction network HMR 2.00 provided in Human Protein Atlas (http://www.metabolicatlas.org/downloads/hmr). The algorithm represents the input network as a bipartite undirected graph where both enzymes and metabolites form nodes and the interactions between them represent edges. The model calculates an enrichment score for each metabolite in the graph based on the normalized differential expression data of its neighbouring enzymes seen in Table S3.

Figure S1 | Metabolic gene expression in TST. Frequency distribution of fold increases and decreases in metabolic gene transcript in TST compared to saline injections.

Figure S2 | Differential metabolic gene expression in tissue and blood. Venn diagram identifying the number of metabolic genes differentially expressed between TST and saline skin injection (red) and between the blood of patients with active TB disease and healthy volunteers (HV) (blue).
Figure S3 | Metabolic genes assigned to individual pathways show both increased and decreased enrichment in the TST. Bar graphs representing gene expression of select metabolic genes in TST and saline groups. Data represented as mean ± SEM from 24 patients with active TB who received a TST (n=16) or saline (n=8) injection. **** p<0.0001 (Mann Whitney test).
### Supplementary Table 1 | KEGG database pathways associated with human metabolism

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Supplementary Table 2 | Genes annotated to human metabolic pathways by KEGG database.
**Supplementary Table 3 | Metabolic genes differentially expressed in TST relative to saline skin injection.**

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Immunometabolism and *M. tuberculosis* challenged human tissue
Supplementary Table 4 | Metabolic genes differentially expressed in blood of patients with active TB disease relative to blood of healthy volunteers.

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Supplementary Table 5 | Metabolic cQTL genes and associated metabolic pathways

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Chapter 8

Metformin alters human host responses to *Mycobacterium tuberculosis* in-vitro and in healthy human subjects

Ekta Lachmandas
Clare Eckold
Julia Bohme
Valerie Koeken
Mardiana Binte Marzuki
Bastiaan Blok
Rob J.W. Arts
Jacqueline Ratter
Corina van den Heuvel
Hazel M. Dockrell
Amit Singhal
Mihai G Netea
Jackie Cliff
Reinout van Crevel

IN PREPARATION
**ABSTRACT**

Metformin, the most widely administered diabetes drug has been proposed as a candidate for host directed therapy for tuberculosis although little is known about its effects on human host responses to *Mycobacterium tuberculosis*.

In this study metformin was added to PBMCs isolated from healthy non-diabetic volunteers in vitro. It enhanced cellular metabolic processes whilst inhibiting the mTOR targets of p70S6K and 4E-BP1. The net result was a decrease in proliferation, cytokine responses and an increase in phagocytosis. In healthy subjects in vivo metformin intake induced significant transcriptional and functional changes. RNAseq and western blot analysis revealed transcriptional down-regulation of oxidative phosphorylation, mTOR signaling and/or type I IFN pathways whilst phagocytosis and ROS genes were increased. Functionally, metformin lowered inflammatory cytokine production of TNF-α (-58%), IFN-γ (-47%) and IL-1β (-20%) whilst inducing anti-mycobacterial processes such as phagocytosis (by 1.5 to 2 fold) and ROS production (+20%). These results show that in humans metformin has a range of potentially beneficial effects on cellular metabolism, immune function and gene-transcription involved in innate host responses to *M. tuberculosis*. This study thus underlines the importance of cellular metabolism for host immunity and supports a role for metformin as host-directed therapy for tuberculosis.
INTRODUCTION

Metformin, a diabetes drug, has been proposed as a candidate for host directed therapy (HDT) in tuberculosis\(^1\). Diabetes itself increases susceptibility to tuberculosis\(^2\) and worsens tuberculosis outcome\(^3\). The mechanisms behind this increase in susceptibility are unclear and a role for diabetes drugs could be envisioned. In particular metformin is anti-inflammatory and inhibits pathways such as mTOR signalling that are important in the host defence to \textit{M. tuberculosis}\(^4\).

Nonetheless, studies in mice and epidemiological data on metformin usage suggest that metformin has beneficial therapeutic effects in the context of tuberculosis disease\(^1\). Proposed mechanisms include an increase in mitochondrial ROS and enhanced killing but so far none of these have been investigated in healthy humans. The mechanism of action behind metformin’s effects are not clearly defined as metformin acts through several pathways including mitochondrial complex I inhibition, an increase in AMP/ATP levels, decreased glucagon and mTOR signaling and an increase in AMPK signaling\(^5\). Lastly it is challenging to study the effects of metformin in patients with diabetes as multiple characteristics of diabetes such as hyperglycaemia, dyslipidaemia, vitamin D deficiency and oxidative stress may all affect cellular immune responses to \textit{M. tuberculosis}\(^6\).

To specifically study the effect of metformin in humans, in this study we first characterised metformin’s effects on human immune responses to \textit{M. tuberculosis} in vitro and then validated these findings in vivo by administration of metformin to healthy volunteers without diabetes. We examined a broad range of transcriptional changes in whole blood and isolated immune cells and functional responses including cytokine production, production of reactive oxygen species (ROS), phagocytosis and \textit{M. tuberculosis} killing.
METHODS

Study subjects
In vitro experiments were performed with peripheral blood mononuclear cells (PBMCs) isolated from buffy coats obtained from healthy volunteers (Sanquin Blood bank, Nijmegen, The Netherlands). As donations were anonymous, no tuberculosis skin tests or IFN-γ release assay was performed. The incidence of tuberculosis in the Dutch population is extremely low (1.5/100,000), and Bacillus Calmette-Guérin (BCG) vaccination is not part of the routine vaccination program. To study the in-vivo effect of metformin, 12 male healthy non-obese volunteers who took no medication and had no kidney function loss or metabolic disorder, were asked to take metformin in increasing doses starting at 1 x 500 mg tablet once a day and ending with 2 x 500 mg tablets twice a day. Blood was drawn at two baseline time points: 1 d before and immediately before metformin intake, and again immediately after, 3 d after and 2 weeks after the last dose of metformin. Experiments were conducted according to the principles expressed in the Declaration of Helsinki. Both for the in-vitro (NL32357.091.10) and healthy volunteers (NL47793.091.14) studies ethical approval was granted by the Arnhem-Nijmegen Ethical Committee. As validation EDTA blood from 10 healthy young subjects given metformin (500 mg day 1-2) increasing to 1000 mg (day 3-8) was examined as part of a pharmacokinetic study (NL53534.091.15).

In-vitro studies
**Cellular isolation and differentiation.** Isolation of PBMCs was performed by differential centrifugation over Ficoll-Paque™ PLUS (GE Healthcare Biosciences). CD14⁺ monocytes were purified from isolated PBMCs using MACS microbeads for positive selection, according to the manufacturer’s instructions (Miltenyi Biotec). To generate macrophages, 3 x 10⁷ PBMCs were incubated at 37°C in petri dishes (Corning) for 1 h. Non-adherent cells were then washed away using warm PBS three times. The remaining adherent monocytes were differentiated into M1 or M2 macrophages in 10% human pooled serum and 5 ng/mL GM-CSF (R&D Systems) or 50 ng/mL M-CSF (R&D Systems) respectively for 6 d. Media containing growth factors and serum were refreshed on day 3 of differentiation. At 6 d post differentiation, adherent macrophages were harvested using Versene Solution (Thermo Fisher Scientific). All cell types were re-suspended in RPMI⁺ (RPMI 1640 (Gibco) supplemented with 10 μg/mL gentamicin (Lonza), 10 mM L-glutamine (Life Technologies), and 10mM pyruvate (Life Technologies). Cells were counted in a Coulter counter (Coulter Electronics) and adjusted to 5 x 10⁶ PBMCs/mL, 1 x 10⁵ CD14⁺ monocytes/mL or 1 x 10⁶ macrophages/mL.
Cytokine production. 100 μL of PBMCs, CD14+ monocytes or 75 μL of M1/M2 macrophages were stimulated in RPMI* with or without 1-5 μg/mL Mycobacterium tuberculosis strain H37Rv (M. tuberculosis) lysate, in the presence or absence of 3-3000 μM Metformin (Sigma-Aldrich) for 4 h, 24 h or with 10% pooled human serum for 7 days. Cell culture supernatants were collected and stored at −20°C. Cytokines in culture supernatants were measured by commercial ELISA kits: interleukin (IL)-1β, tumour necrosis factor (TNF)-α, IL-17A, IL-22 (R&D Systems) and IL-6, interferon gamma (IFN-γ) and IL-10 (Sanquin).

Cellular proliferation. Proliferation of PBMCs was measured using the CFSE kit (BioLegend) according to the instructions described by the manufacturer. Briefly, PBMCs were re-suspended at a density of 10 x 10⁶ cells/mL in PBS and labelled by adding CFSE in a 1:1 ratio at a final concentration of 1.25 μM. The suspension was mixed gently and incubated for 5 min at 37°C. An equal volume of 100% human pooled serum was added and incubated for 3 min at room temperature. Cells were washed twice in RPMI supplemented with 10% human pooled serum and re-suspended to a concentration of 5 x 10⁶ PBMCs/mL in RPMI*. An unlabelled cell fraction and a labelled cell fraction were measured by flow cytometry on day 0 to determine staining efficacy. Labelled cells were stimulated for 6 d with M. tuberculosis lysate in the presence or absence of 300 μM metformin. On day 6, cells were stained with anti-CD4 (PE-Cy5 conjugated, ITK Diagnostics BV) and measured by flow cytometry. The percentage of proliferated CD4+ cells was calculated as percentage of all CD4+ cells.

Metabolic measurements. Lactate was measured from stored cell culture supernatants using a coupled enzymatic assay in which lactate was oxidised and the resulting H₂O₂ was coupled to the conversion of Amplex® Red reagent to fluorescent resorufin by HRP (horseradish peroxidase). Measurement of the NAD+/NADH redox ratio was adapted from Zhu et al. Glucose consumption was measured according to the manufacturer’s instructions using the Amplex® Red Glucose/Glucose Oxidase Assay Kit (Life Technologies).

Western Blot Measurements. Pellets of 5 x 10⁶ PBMCs per condition were lysed in 100 μL lysis buffer (1M Tris pH 7.4, 0.5M EDTA, 5M NaCl, 10% ND40, 0.5M NaF, 2.5% sodium deoxycholate, PhosSTOP (Roche) and cOmplete (Roche)). The cell homogenate was frozen, thawed and processed for Western blot analysis according to the manufacturer’s instructions. Western blotting was carried out using Mini-PROTEAN TGX precast Gels (Bio-Rad). Proteins were transferred using the Trans-Blot® Turbo™ system (Bio-Rad) according to the manufacturer’s instructions. Blots were incubated overnight at 4°C with actin at 1:1000, phospho-AMPK (T172) (p-AMPK) at 1:500, phospho p70 S6K (T389) (p-p70 S6K) at 1:500, phospho-4EBP1 (T37/460) (p-4EBP1) at 1:1000, phospho-P38 (T180/Y182) (p-P38) at 1:1000, total-P38 at 1:000 or phospho-AKT (S473) (p-AKT) at 1:1000. Actin was bought from Sigma and all other
antibodies from Cell Signalling. Secondary antibody used was swine anti-rabbit at 1:5000 (Dako). SuperSignal West Femto Substrate (Thermo Fisher Scientific) or ECL (Bio-Rad) were used for visualisation of proteins.

**Reactive Oxygen Species Measurements.** 100 μL of whole blood diluted 100x in Hanks’ buffered salt solution (HBSS) or 50 μL of a total of 2.5 x 10^6 PBMCs together with 50 μL of HBSS was added in quadruplicate to each well of a white 96-well assay plate (Corning). Cells were incubated with 50 μL of 1 mg/mL serum-opsonized zymosan, 50 μL of 10 μg/mL serum-opsonised *M. tuberculosis* lysate or 50 μL of 100% human pooled serum as control. 50 μL of 145 μg/mL luminol (Sigma) was added, and chemiluminescence was measured every 142 s for 1 h. Opsonized zymosan particles were prepared by incubation of zymosan derived from *Saccharomyces cerevisiae* (Sigma-Aldrich) in pooled human serum for 30 min at 37°C, after which the particles were washed twice in PBS and re-suspended in PBS. Opsonised *M. tuberculosis* lysate was prepared by incubation of lysate in 100% human pooled serum for 1 h prior to making aliquots and freezing.

**Phagocytosis.** pHrodo® Green Zymosan Bioparticles® Conjugate (Thermo Fisher Scientific) was used to measure the rate of phagocytosis. The pH-sensitive molecular probes are almost non-fluorescent in a neutral environment, but the dye lights up when the pH decreases in the lysosome. So the amount of emitted fluorescence is a relative measure for the rate of phagocytosis. One vial of pHrodo particles was dissolved in 2 mL RPMI and sonicated for 5 min. For *in-vitro* measurements, 5 x 10^5 PBMCs were incubated for 24 h at 37°C in a flat bottom black plate with RPMI or 1000 μM metformin. The supernatant was removed and 100 μl RPMI (cells only control) or pH rodo suspension was added to each well in duplicate and incubated for 2 h in a non-CO2 elevated incubator at 37°C before measuring fluorescence. For the trial volunteers, peripheral blood leukocytes were isolated by lysis of erythrocytes using hypotonic lysis buffer (155 mM NH4Cl, 10 mM KHCO3). After isolation, the cells were rested for 30 min at 37°C in a black 96-wells plate, after which RPMI (cells only control) or pH rodo suspension was added to each well in duplicate and incubated for 2 h in a non-CO2 elevated incubator at 37°C before measuring fluorescence. Fluorescence was measured at an excitation of 486 nm and an emission rate of 528 nm.

**M. tuberculosis cellular infection.** Frozen mycobacteria, H37Rv, were thawed, washed and re-suspended in antibiotic-free RPMI 1640 with 10% fetal bovine serum (FBS) and were used to infect 3 x 10^6 PBMCs in 15 mL falcon tubes with a multiplicity of infection (MOI) of 5. The infected cells were incubated at 37°C with 5% CO2 for 3 h. After this time, cells were washed two times with antibiotic-free medium by centrifuging at 800 rpm. The infected cells were counted and seeded in triplicate for 3, 24, or 48 h. At predetermined time points after infection, the infected cells were washed once with PBS and then lysed with 200 mL.
of PBS with 1% SDS. Various dilutions of this lysate were plated on Middlebrook 7H11 agar supplemented with 10% oleic acid–albumin–dextrose–catalase (OADC, Difco Laboratories), in triplicate. Agar plates were incubated at 37°C for 3 weeks, after which colonies were counted visually. CFUs obtained from two or three dilutions were used to calculate the total number of CFU per mL.

**Cellular Viability.** To examine early or late apoptosis, 5 x 10^5 treated PBMCs were washed with PBS, re-suspended in 200 μL of RPMI and incubated on ice with 1 μL of Annexin V-FITC (FITC, Biovision) for 15 min followed by a 5 min incubation with 1.5 μL of propidium-iodide (PI, Sigma Aldrich). The relative level of apoptotic cells was detected by flow cytometry within 1 h, using a FC500 flow cytometer (Beckman Coulter) and data were analysed using Kaluza 1.3 software (Beckman Coulter).

**Transcriptomics**

Whole blood was captured in Paxgene tubes and stimulated PBMCs or CD14^+ monocytes were lysed in TRIzol reagent (Invitrogen) or RNAprotect (Qiagen) and stored at −80°C until RNA isolation was performed. RNA was isolated from purified CD14^+ cells using TRIzol reagent (Invitrogen), from whole blood samples using the PAXgene blood miRNA kit (Qiagen) and from cultured PBMC samples using the RNeasy Mini Kit (Qiagen) following the manufacturers’ protocols. RNA was transcribed into complementary DNA by reverse-transcription using either the iScript cDNA synthesis kit (Biorad) or the Superscript IV VILO (Invitrogen). Primer sequences used for Quantitative real-time PCR (qPCR) are listed in Supplementary Table 1. Power SYBR Green PCR Master Mix (Applied Biosystems, Life technologies) was used for qPCR in an AB Step one plus or 7500 Fast real-time PCR system (Applied Biosystems) or the CFX384 Real-Time PCR Detection System (Biorad). qPCR data were normalized to the housekeeping gene human β2M or HuP0.

For RNA-sequencing, RNA was quantified using Nanodrop ND1000 spectrophotometer and quality assured using an Agilent 2100 Bioanalyzer. RNA from the whole blood Paxgene samples was depleted of globin transcripts using GLOBINclear™ (Invitrogen). Whole blood samples were processed using the Ribo-Zero TruSeq stranded total RNA library preparation method (Illumina) whereas cultured PBMC RNA samples were processed using the TruSeq stranded mRNA library prep kit (Illumina). All samples were sequenced on a NextSeq500, generating ~36–45M million 43bp paired-end reads per sample. FASTQ sequence files were aligned to the human genome version Human_g1k_v37 using STARAligner^9 and aligned reads which overlapped with 63,677 genome features were counted using HTSeq-count with annotation version Homo_sapiens.GRCh37.75.gtf^10. Differentially expressed genes were calculated using the DESeq2 package^11 in Bioconductor, with false discovery rate correction applied for multiple testing. Gene set analyses were performed using the Stouffer
method in the Piano R package\textsuperscript{12} with the MSigDB Hallmark gene sets database\textsuperscript{13}, as well as the KEGG pathways database. The RNA Seq study was approved by the LSHTM Research Ethics Committee (\#11968).
RESULTS

In-vitro effects of metformin on cellular metabolism and cytokine production. When added to PBMCs metformin decreased the levels of the downstream mTOR targets, phospho-p70S6K and phospho-4EBP1 whilst increasing its known molecular target: AMPK (Fig. 1a). Both lactate production and glucose consumption were increased in M. tuberculosis stimulated PBMCs in the presence of metformin (Fig. 1b and 1c) whilst the NAD+/NADH ratio was decreased (Fig. 1d).

![Figure 1](image-url)

**Figure 1 |** Metformin alters mTOR signaling axis whilst maintaining glucose regulatory effects. (a) Western blots of PBMCs stimulated with M. tuberculosis lysate for 2 h in the presence or absence of 1000 µM metformin (Met). Data are from two individual experiments. (b) NAD+/NADH fold change of Mtb stimulated vs unstimulated PBMCs in the presence or absence of 1000 µM metformin for 24 h, 48 h, or 7 d. Data (mean ± s.e.m.) are from two experiments with a total of six donors. (c,d) Lactate production and glucose consumption from PBMCs stimulated with M. tuberculosis lysate in the presence or absence of 1000 µM metformin for 24 h, 48 h, or 7 d. Data (mean ± s.e.m.) are from three experiments with a total of nine donors. (d) * p<0.05, ** p<0.01 (Wilcoxon matched-pairs signed rank test).
At both therapeutic (10 – 220 µM) and experimental concentrations metformin showed clear effects on cytokine production. It significantly decreased *M. tuberculosis* induced TNF-α, IL-1β, IFN-γ and IL-17 production from PBMCs (Fig. 2a), IL-1β, IL-6 and IL-10 from M1 and M2 monocyte derived human macrophages (Fig. 2b) and TNF-α, IL-1 β and IL-10 from CD14+ monocytes (Supplementary Fig. 1a). At a transcriptional level, metformin inhibited expression of IL-18, IL-23 p19 and TGF- β1 (Fig. 2c). The minimal effect of metformin on cellular proliferation (Fig. 2d) is unlikely to account for the strong effects on cytokine production (Figure 2e). Metformin at the doses tested also had no significant effect on cellular viability (Supplementary Fig. 1b).

**Effect of metformin on immune phenotype and transcriptomics in healthy subjects**

We next examined the effect of metformin in healthy subjects. Blood was drawn at several time-points before and after metformin intake (Fig. 3a). We first examined changes in AMPK activation in PBMCs (Fig. 3b and Supplementary Fig. 2a). As expected, phospho-AMPK was significantly increased in both un-stimulated and *M. tuberculosis* lysate stimulated conditions after metformin intake (Td6 vs Td0) (Fig. 3c and 3d).

Next, RNA-Seq analysis of whole blood samples collected before and after 5 days of metformin intake was performed. Using an unbiased approach, no significant effects were seen on individual genes (Supplementary Fig. 2b), but at a biological pathway level metformin induced consistent changes (Fig. 3e). Significant down-regulation of oxidative phosphorylation (OXPHOS) and ribosome pathways whereas significant up-regulation of endocytosis/phagocytosis, MAPK and chemokine signaling pathways was observed. Similar results were obtained when a second analysis platform, GAGE, was used (data not shown).

In PBMCs isolated from subjects before and after (Td6) metformin intake, metformin significantly affected gene transcription, both in stimulated and unstimulated cells, with approximately 800 genes differentially expressed (Supplementary Fig. 2c). In gene set analysis of unstimulated PBMC, metformin led to upregulation of genes involved in mitosis and down-regulation of genes involved in OXPHOS, adipogenesis and myc targets (Fig. 3f). In *M. tuberculosis* stimulated cells, metformin suppressed genes involved in cytokine signalling such as IFN-α, IFN-γ and TNF-α, OXPHOS and mTORC related genes (Figure 3f).
Figure 2 | Metformin affects the cytokine landscape of human cells responding to *M. tuberculosis* stimulation. Cytokine production from (a) human PBMCs stimulated with *M. tuberculosis* lysate in the presence or absence of 3–3000 µM of metformin after 24 h (TNF-α, IL-6, IL-1β and IL-10) or after 7 d (IFN-γ, IL-17 or IL-22) in the presence of 10% pooled human serum and (b) from M1 and M2 macrophages stimulated *M. tuberculosis* lysate in the presence or absence of 300 & 3000 µM of metformin after 24 h. (c) Gene expression of cytokines from CD14+ monocytes stimulated with *M. tuberculosis* lysate in the presence or absence of 3000 µM of metformin after 4 h (IL-18 and TGF-β1) or after 24 h (IL-23 p19 subunit and IL-12 p35 subunit). (d) % of CD4+ T cell proliferation in PBMCs stimulated with *M. tuberculosis* lysate in the presence or absence of 300 µM metformin for 6 d. CFSE dye was used to track generations. (e) Radial graph is representative of fold changes between *M. tuberculosis* lysate stimulated in the presence of 3000 µM metformin relative to *M. tuberculosis* lysate stimulation alone. The axis represents the degree of fold change. Less than 1 is a decrease in responses and is indicated by projection towards the centre of the radius. For a-c and e all data (mean ± s.e.m.) are from three experiments with a total of six to thirteen donors. For d data are (mean ± s.e.m.) from four experiments with a total of seven donors. *p<0.05, **p<0.01 (Wilcoxon matched-pairs signed rank test for 1a–c and Paired t test for 1d).
Figure 3 | Global effects of metformin in healthy human volunteers. (a) Healthy volunteers (n = 11) received an increasing dose of metformin for five consecutive days. Blood was drawn twice pre- (Td0) and several times post-metformin treatment. (b) Western blot analysis of p-AMPK levels in lysates of PBMCs stimulated for 2 h RPMI (-) or *M. tuberculosis* lysate (+) from healthy volunteers before and after metformin intake. (c) Quantitative relative band intensity analysis of p-AMPK between pre- (Td0) and post-metformin (Td6) periods for RPMI and *M. tuberculosis* lysate stimulation. * p<0.05, ** p<0.01 (Paired t test). (d) Fold change in p-AMPK levels between pre-(Td0) and post-metformin (Td6) periods for RPMI and *M. tuberculosis* lysate stimulation. * p<0.05, ** p<0.01 (Paired t test). All western blot data (mean ± s.e.m.) are representative of a total of eight donors presented in figure 3b and supplementary figure 2a. (e) Gene set analysis showing KEGG pathways which were differentially expressed in ex vivo blood samples following metformin administration. The bar length indicates the magnitude of the change of the gene set. Data were analyzed using the Piano R package, and pathways with adjusted P<0.01 are shown. (f) Hallmark gene set enrichment and network analysis, showing gene sets up- (red) or down- (blue) regulated following metformin administration in PBMCs in either resting state or stimulated with *M. tuberculosis* lysate for 4 h.
Each gene ontology (GO) group in the identified gene sets was investigated, and the “response to type 1 interferon” GO set showed the most markedly reduced expression following metformin administration in *M. tuberculosis* lysate-stimulated cultures (Supplementary Fig. 2D). Within this GO, the expression of eight genes (Interferon-induced protein with tetratricopeptide repeats (IFIT) 1, IFIT 2 and IFIT 3, 2’-5’-oligoadenylate synthase (OAS) 1, OAS2 and OAS3, MX dynamin like GTPase (MX) 1 and radical S-adenosyl methionine domain containing 2 (RSAD2)) was more than two-fold reduced following metformin administration in cells stimulated with *M. tuberculosis* for 4 hours (Fig. 4a.). Thus the type 1 interferon response was blocked by metformin. This effect was diminished by 24 hours as shown by qRT-PCR (Fig. 4a). Additionally, after metformin intake (Td6) a significant decrease in TNF-α, IL-1β, IL-6, IFN-γ and IL-17 release in response to stimulation was found (Fig. 4b). This effect was sustained significantly for TNF-α and insignificantly for IL-1β, IFN-γ and IL-22 up to 21 days post metformin intake.

The MAPK, AKT and mTOR pathways are known to strongly influence cytokine production. So we measured the levels of p-P38 and total-P38 (Fig. 4c and Supplementary Fig. 3a), a member of the MAPK family, p-AKT and p-4EBP1, a representative of the mTOR pathway (Fig. 4d and Supplementary Fig. 3b), in cell lysates taken pre- and post-metformin intake. An overall decrease in all three targets were observed. Quantitative band intensity analysis showed that the ratio of p-P38 to total-P38 and the levels of p-AKT were significantly reduced by metformin (Fig. 4e and 4f). Similarly, metformin reduced p-4EBP1 levels however significance was not achieved (Fig. 4g). Effects at an individual level can be seen in Supplementary Figures 3c (p-P38/total-P38), 3d (p-AKT) and 3e (p-4EBP1).

**Effects of metformin on cellular phenotype**

Metformin showed mixed effects on the number, relative distribution and activation of immune cells. In whole blood, metformin led to a transient increase in total white blood cells (WBC) and neutrophils (Fig. 5a), but the relative percentage of cell types (monocytes, lymphocytes, neutrophils, eosinophils and basophils) did not change over time (Fig. 5b). In PBMCs, we observed a sustained increase in the relative percentage of monocytes and a decrease in the proportion of lymphocytes (Fig 5c).
Figure 4: Metformin intake in healthy volunteers affects cytokine production via P38 and AKT inhibition.
Figure 4 | Metformin intake in healthy volunteers affects cytokine production via P38 and AKT inhibition. (a) Expression of eight genes in the “response to type 1 interferon” Gene Ontology group in PBMC stimulated with *M. tuberculosis* lysate in vitro for 4 or 24 h, before and after in vivo metformin administration in 11 healthy volunteers. Expression measured by RNA-Seq (4hr) and qRT-PCR (4 and 24hr). (b) Cytokine production from isolated PBMCs stimulated with *M. tuberculosis* lysate 24 h (TNF-α, IL-6, IL-1β and IL-10) or after 7 d (IFN-γ, IL-17 or IL-22) in the presence of 10% pooled human serum before and after metformin intake. (c) Western blot analysis of p-38 and Total p38 and (d) p-AKT and p-4EBP1 levels in lysates of PBMCs stimulated for 2 h RPMI (-) or *M. tuberculosis* lysate (+) from healthy volunteers before and after metformin intake. Data are representative of four of eight measured donors from the trial. (e-g) Fold change in p-38/Total p38 levels, p-AKT or p-4EBP1 between pre- (Td0) and post-metformin (Td6) periods for RPMI and *M. tuberculosis* lysate stimulation. * p<0.05, ** p<0.01 (Paired t test.). All western blot data (mean ± s.e.m.) are representative of a total of eight donors presented in figure 4c or 4d and supplementary figures 3a or 3b respectively.

Figure 5 | Metformin intake in healthy volunteers alters the blood cellular composition landscape. Analysis of leukocyte counts plotted (a) as raw cell counts for whole blood, (b) as percentage of total counts for whole blood and (c) as percentage of total counts for isolated PBMCs.
The effect of metformin on ROS production, phagocytosis and mycobacterial killing.

Next we examined the effect of metformin on another host defence mechanism namely, ROS production. In whole blood a strong and significant increase in ROS production was detected immediately post metformin treatment (Fig. 6a), spontaneously and upon stimulation with *M. tuberculosis* lysate and zymosan. No increase in ROS was observed from PBMCs (Supplementary Fig. 4a). In line with increased ROS production in whole blood, genes involved in ROS production such as NADPH Oxidase 2 (*cybb*), p22-PHOX (*cyba*), RAC1 and particularly for ROS production in neutrophils p47-PHOX (*ncf1*), p67-PHOX (*ncf2*) and p40-PHOX (*ncf4*) were strongly upregulated in blood after metformin intake (Fig. 6b).

Another key host defence strategy is the phagocytosis of mycobacteria. Whole blood RNAseq analysis revealed that metformin upregulated genes involved in endocytosis such as receptors (RTKs and GPCR), regulators of clathrin-mediated pit formation (AP2) and clathrin uncoating (Hsp70) and regulators of intracellular vesicular trafficking (Arfs, ArgGAPs and ArfGEFs) (Figure 6c). To validate these findings, we examined phagocytosis in a second group of healthy subjects taking metformin. In line with the transcriptional findings, metformin increased phagocytosis of zymosan labelled beads in whole blood (Fig. 6d). In vitro pretreated PBMCs also showed upregulated phagocytosis (Supplementary Fig. 4b).

Finally, mycobacterial viability was examined in cryo-preserved PBMCs isolated from subjects before and after metformin intake. No significant difference was seen in mycobacterial outgrowth at different time points after infection (Fig. 6e and Supplementary Figure 4c).
Figure 6 | Metformin intake in healthy volunteers increases anti-mycobacterial defence mechanisms. (a) ROS production as measured by luminol-reaction from whole blood from pre- and post-metformin treated volunteers unstimulated (RPMI) or stimulated with M. tuberculosis lysate (Mtb) or zymosan. Data are representative of 11 individual donors. Bars representing the fold-change of Td6, Td9 or Td21 over TdB for each individual donor are superimposed with grey dots representing the mean ± s.e.m. (b) Expression of six genes encoding key NADPH oxidase proteins for ROS production were assessed in ex vivo blood by RNA-Seq before and after administration of metformin in the healthy volunteers. * p<0.05, ** p<0.01 (Wilcoxon matched-pairs signed rank test). (c) Representation of KEGG endocytosis pathway. Net difference in gene expression of phagocytosis related genes in whole blood before and after metformin intake. Blue indicates a down-regulation whilst red indicates an up-regulation. (d) Net phagocytosis of pHrodo conjugates in a validation trial where healthy volunteers were given metformin for eight days and blood was drawn before (Td1) and after the trial (Td8). Lysed blood was incubated with the pH rodo suspension for 2 h in a non-CO2 elevated incubator at 37°C before measuring fluorescence. (e) Yield of colony forming units (CFUs) of H37Rv from infected PBMCs obtained before and after metformin intake.
DISCUSSION

Metformin is the most widely used diabetes drug worldwide. It has been proposed for adjunctive TB treatment although its effects in humans are not wholly characterised. In this study we examine for the first time the detailed effects of metformin on the immune responses of healthy individuals before and after metformin intake. We found significant changes in several host defence mechanisms. Specifically, a decrease in cytokine production and an increase in ROS production and phagocytic capacity was found. These changes were mediated by several mechanisms including inhibition of the type 1 interferon pathway, a decrease in AKT and P38 signalling and an increase in AMPK signalling.

Both in vitro and in vivo a strong effect of metformin on inflammatory cytokine signalling was observed. Notably metformin inhibited the type 1 interferon response by blocking the interferon-stimulated genes IFIT1, IFIT2 and IFIT3 which, amongst other activities, regulate inflammatory cytokine mRNA stability, cell proliferation and apoptosis. Neutrophil driven type 1 interferon signalling, including upregulated IFIT1, IFIT2, IFIT3, OAS1, OAS2, OAS3, RSAD2 and MX1 gene expression in blood, has been identified as a signature of active tuberculosis disease. Inhibiting this pathway using an arachidonic acid metabolism modulator namely, zileuton, has been shown to have protective effects against tuberculosis in mice. Our data show that metformin too has the capacity to down-regulate the type-1 interferon pathway and thus sheds light onto a novel mechanism through which metformin's protective effects might be mediated.

ROS production and phagocytosis were increased by metformin. The increase in ROS did not correlate with an increase in white blood cell counts or neutrophil counts (Supplementary Fig. 5a and 5b) whereas phagocytosis correlated with an increase in WBC counts but not neutrophil counts (Supplementary Fig. 5c). The lack of strong correlations suggests that the observed effects are intrinsically mediated by metformin. This is supported by the accompanying transcriptional changes observed in both ROS and phagocytosis related genes and the increase in phagocytosis induced by metformin in vitro. Mechanistically, AMPK activation has been linked to phagocytosis activity as pharmacologic or genetic ablation of AMPK subunits negatively influences phagocytosis. As this is the first exploratory study of the effects of metformin on immune function in vivo it would be of future interest to examine the phagocytic capacity of antigen presenting cells such as macrophages and dendritic cells post metformin treatment.

In this study, the mycobacterial killing capacity of PBMCs before and after metformin treatment remained unchanged. This is in contrast to a recent publication which showed that human monocyte derived macrophages pre-treated with metformin displayed
enhances mycobacterial killing\textsuperscript{1}. There are several explanations for this discordance in results. Firstly, PBMCs before and after metformin intake were cryo-preserved and later thawed for infection assays. This process may have resulted in a loss of the ‘metformin’ phenotype. Secondly, metformin was not directly present in culture, which suggests that activation of the mycobacterial killing machinery may require near constant metformin exposure. Lastly, it is likely that a different cell type such as neutrophils, macrophages or a systemic model encompassing all the above changes in cytokine, phagocytosis and ROS production is needed to see the proposed anti-mycobacterial effects of metformin.

Metformin is put forward as a candidate for host-directed therapy in TB but some caution is advised. In contrast to TB, in a model of candidemia metformin resulted in increased lethality\textsuperscript{24}. Secondly, it is still unknown if simultaneous administration of tuberculosis drugs and metformin will lead to harmful drug-drug interactions or more toxicity. Metformin is a substrate for human organic cation transporters (OCT) and multidrug and toxin extrusion proteins (MATEs)\textsuperscript{25}. Rifampicin, a first-line anti-tuberculosis drug, increases the expression of OCT1 and increases metformin transport into the liver leading to an enhanced glucose-lowering effect in healthy individuals\textsuperscript{26}. Rifampicin also induces OCT2 which increases metformin renal clearance. Ethambutol – another first line drug - potently inhibits OCT1, OCT2 and more weakly OCT3 suggesting that significant drug-drug interactions may take place in situations of co-administration\textsuperscript{27}. Interestingly an in vitro study specifically examining the drug-drug interactions between metformin and tuberculosis drugs found that only moxifloxacin was a potent inhibitor of metformin transport\textsuperscript{25}. In vivo human studies exploring the relationship between tuberculosis drugs and metformin will be key in advancing metformin as adjunctive tuberculosis therapy.

Besides metformin, other anti-diabetes drugs may also impact host response to \textit{M. tuberculosis}. Glibenclamide, a sulfonylurea derivate, has been reported to reduce mortality due to melllioidosis by reducing the hyper-inflammation that leads to sepsis \textsuperscript{28, 29}. In vitro, glibenclamide reduces pro-inflammatory cytokine production in response to \textit{M. tuberculosis} stimulation (Supplementary Fig. 5d). Similarly, thiazolidinediones, another class of diabetes drugs, have been linked with reduced risk for development of sepsis\textsuperscript{30}.

In summary we have learned that metformin dampens the pathological inflammatory responses associated with TB lung lesions and disease severity. Simultaneously, it enhances anti-mycobacterial processes such as ROS and phagocytosis which promote bacterial clearance. These data thus provide in vivo and human based evidence to support metformin as a candidate for adjunctive therapy in tuberculosis and to be tested in fittingly designed clinical trials.
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REFERENCES


**SUPPLEMENTARY MATERIAL**

![Supplementary Figure 1](image)

**Supplementary Figure 1 | Metformin affects the cytokine landscape of human cells responding to *M. tuberculosis* stimulation.** Cytokine production from (a) CD14+ monocytes stimulated with *M. tuberculosis* lysate in the presence or absence of 3000 µM of metformin after 24 h (TNF-α, IL-6, IL-1β and IL-10). All data (mean ± s.e.m.) are from two to three experiments with a total of six donors. * p<0.05, ** p<0.01 (Wilcoxon matched-pairs signed rank test). (b) PBMCs were pre-incubated with stimulated with *M. tuberculosis* lysate in the presence or absence of 3 - 3000 µM of metformin for 1, 3 or 7 d. Cells were harvested and stained with an Annexin-V / PI stain to determine levels of cell death. Data are represented as % means ± SEM (n = 4–6).
Supplementary Figure 2 | Differential gene expression following metformin administration.
(a) Western blot analysis of p-AMPK and Actin in lysates of PBMCs stimulated for 2 h RPMI (-) or *M. tuberculosis* lysate (+) from healthy volunteers before and after metformin intake. Data are representative of the remaining four of eight donors in the trial presented in figure 3b. (b) Ex vivo paxgene RNA samples from 11 healthy donors were analysed before and after metformin administration for 5 days. (c) PBMC samples from the 11 donors taken before or after 5 d metformin were cultured in the absence or presence of *M. tuberculosis* lysate for four hours: the data for both conditions are combined. For (b) and (c) data shown are mean expression for each individual gene, and genes shown in purple were significantly differently expressed (FDR<0.05). (d) Adjusted p values for differential expression of genes in the GO set ‘type 1 interferon’ compared to all the adjusted p values, and to the ‘GO regulation of dopamine metabolic processes’ pathway.
Supplementary Figure 3 | Activation levels of p-38, p-AKT and p-4EBP1 before and after metformin intake. Western blot analysis of (a) p-38 and Total p38 and (b) p-AKT and p-4EBP1 levels in lysates of PBMCs stimulated for 2 h RPMI (-) or M. tuberculosis lysate (+) from healthy volunteers before and after metformin intake. Data are representative of the remaining four of eight donors from the trial. Fold change in (c) p-p38/Total p38 (d) p-AKT and (e) p-4EBP1 levels between pre- (Td0) and post-metformin (Td6) periods for RPMI and M. tuberculosis lysate stimulation. * p<0.05, ** p<0.01 (Paired t test). All western blot data (mean ± s.e.m.) are representative of a total of eight donors presented in figures 4c and 4d and supplementary figures 3a and 3b respectively.
Supplementary Figure 4 | Effects of metformin on host defence mechanisms. (a) ROS production as measured by luminol-reaction from PBMCs from pre- and post-metformin treated volunteers unstimulated (RPMI) or stimulated with *M. tuberculosis* lysate (Mtb) or zymosan. (b) Net phagocytosis of pHrodo conjugates by PBMCs untreated (RPMI) or treated with metformin for 24 h prior to incubation with pHrodo conjugates for 2 hr. (c) Percentage change (%) in colony forming units (CFU) between 24 h or 48 h and 3 h of infection of PBMCs from pre- and post-metformin treated volunteers infected with mycobacteria.
Supplementary Figure 5 | Discussion. Pearson correlation of (a) ROS with white blood cell counts (WBC), (b) ROS with neutrophil counts or (c) Phagocytosis with WBC or neutrophil counts. Significance and R coefficient indicated in table below each graph. ** p<0.01 (d) Production of IL-1β or TNF-α in response to M. tuberculosis lysate in the presence of a dose range (ng/mL) of three diabetes drugs, namely Glibenclamide (Glib), Glimeperide (Glim) or Pioglitazone.
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Chapter 9

Summary
General Discussion &
Future Perspectives
PART I

The impact of diabetes on susceptibility to tuberculosis
SUMMARY

Tuberculosis has co-evolved with humans and its eradication has proven to be a formidable challenge. Roughly 15% of all TB cases are attributable to diabetes\(^1\), with diabetes itself projected to rise by 55% in the next 25 years\(^2\). As diabetes threatens global tuberculosis control, a better understanding of the mechanisms underlying the effects of diabetes on tuberculosis susceptibility and outcome is greatly needed.

Patients with diabetes mellitus type II are characterised by several physiological changes that include but are not limited to hyperglycaemia, hyperinsulinemia, dyslipidaemia, hyperuricemia and an altered microbiota. It is not known which of these factors influence susceptibility to tuberculosis. To this end I investigated the effects of three factors, namely in vitro hyperglycaemia, in vivo hyperglycaemia, and products of the gut microbiome which may contribute to increased susceptibility of diabetes patients to TB.

Hyperglycaemia has often been correlated with changes in the immune system\(^3, 4, 5, 6\) but few studies exploring the role of hyperglycaemia in TB infection have been performed. Therefore, in Chapter 2 I examined for the first time whether high glucose concentrations could affect host defences to \(M.\) tuberculosis in vitro. A mild increase in pro-inflammatory cytokines when using high glucose cultures was observed. However, phagocytosis and the ability of macrophages to control tuberculosis infection was unaffected. These results suggest that short term hyperglycaemic cultures do not strongly affect the immune response and clearance of mycobacteria. Following this study, we questioned whether chronic, rather than acute hyperglycaemia could modulate host defences to mycobacteria. Therefore, in Chapter 3 we recruited a cohort of 20 T1DM males with chronic hyperglycaemia, no medication except regular insulin, and no serious secondary complications. In response to \(M.\) tuberculosis stimulation we found a defect in the IL-1β, IL-6 and IFN-γ signalling pathways in T1DM patients compared to matched healthy controls. These changes were not explained by physiological factors in serum such as plasma glucose levels, changes in cellular metabolism or changes in pattern recognition receptors.

Looking at susceptibility from another angle we turned to an emerging area of research in diabetes: the study of the gut microbiome. Cohort studies have shown that patients with T2DM have a distinct change in their gut microbiota\(^7, 8\). In particular, profound changes in their composition of SCFA have been described where physiological levels of butyrate-producing bacteria such as \(Roseburia\)\(^7, 8\) and \(Faecalibacterium prausnitzii\)\(^9\) are decreased whereas \(Bacteroides\)\(^2, 9\) and \(Escherichia coli\) are increased\(^7\). To determine whether SCFA could modulate host responses to mycobacteria in Chapter 4 we examined the effects of butyrate on cytokine production in response to \(M.\) tuberculosis stimulation. We found that
minute but physiologically relevant plasma concentrations of butyrate lowered levels of inflammatory cytokines via transcriptional upregulation of IL-10. Subsequent blockade of IL-10 inhibited the anti-inflammatory effects of butyrate.
DISCUSSION

With the help of these and other recently published in vitro and in vivo studies, we can begin to answer the following question: Are there specific factors or mechanisms that increase susceptibility of diabetes patients to tuberculosis?

The role of hyperglycaemia on cytokine responses to *M. tuberculous*

Epidemiological studies have provided evidence for a strong link between hyperglycaemia (HbA1c > 7%), glycaemia control and the risk of TB. In fact, one of the studies demonstrated a linear relationship between fasting plasma glucose and risk of tuberculosis. In line with this an in vivo mouse model of TB and T2DM showed that mice with chronic but not acute diabetes were susceptible to TB infection. This confirms our observations that in vitro acute hyperglycaemia does not affect the immune response to tuberculosis. In vivo we showed that PBMCs from T1DM patients produced less IL-1β, IL-6 and IFN-γ, although these changes were not correlated with HbA1c or plasma glucose levels. This lack of correlation with hyperglycaemia prompted us to test correlations with insulin or T1DM duration, however no correlations were found. Other factors such as cholesterol and triglycerides levels were unchanged. A limitation of our study from Chapter 3 is represented by the small cohort (n= 20) compared to the large epidemiological cohort (n= >120,000) described above. Therefore, subtle associations between glucose and cytokine responses may not be detectable.

Interestingly, a series of studies by Kumar et al. show that patients with both active TB and T2DM produce more Th1 and Th17 cytokine, whereas patients with both latent TB (LTB) and T2DM produce less pro-inflammatory cytokines. Cytokine changes in the Kumar et al. studies correlated with hyperglycaemia levels only during concomitant TB-DM but not LTB-DM. This suggests that correlations with hyperglycaemia are only evident once disease is established.

If this is true, how would hyperglycaemia alter host immune response mechanisms?

A variety of studies suggest that epigenetics may be a root cause. Firstly, glucose has been shown to cause persistent epigenetic changes in the promoter of the NF-kB subunit p65 leading to gene expression changes for pro-inflammatory cytokines. Secondly, a study by Martinez et al. has shown that T-cells from hyperglycaemic mice have more condensed chromatin compared to healthy mice which supports transcription and pro-inflammatory cytokine production. Lastly in support of these studies, Prada-Medina et al. have shown that TB-DM patients are characterised by transcriptional changes in epigenetic regulatory pathways. Thus, a future study of the epigenome of naïve and mycobacteria stimulated
cells from patients with TB, TB-DM, DM only and healthy controls would allow us to delineate the possible role of epigenetics and hyperglycaemia in the enhanced susceptibility of DM patients to TB.

The gut microbiome

The composition of the gut microbiota can influence host immune responses. Patients with diabetes have a decrease in the number of butyrate producing bacteria\(^7,8\). Butyrate has been shown to reduce secretion of pro-inflammatory cytokines. In diabetes a decrease in butyrate suggests that this control system is tipped in favour of inflammation. Our study shows that at physiologically relevant concentrations butyrate can ameliorate the immune response to TB, thus bringing potential therapeutic value to butyrate supplementation. In the case of concomitant TB-DM, butyrate could be used to curb excessive inflammation and pathology. Butyrate has also been shown to improve insulin sensitivity and suppress weight gain in obese and diabetic mice\(^19,20\). Thus, if excessive inflammation contributes to susceptibility then attenuation of inflammation using butyrate may improve outcome and treatment efficacy in patients with both TB and DM. Alternatively, drugs or small molecules with similar properties to butyrate should be explored.

Potential other mechanisms

Another common characteristic of T2DM is high triglyceride or lipid levels. Peyron et al. have shown that virulent mycobacteria can induce the formation of ‘foamy’ lipid laden macrophages that have a reduced capacity to phagocytose or kill mycobacteria\(^21\). In their study *M. tuberculosis*-containing phagosomes merged with lipid bodies within the cell suggesting that excess fats may represent a reservoir of fuel for survival and ultimately another mechanism for susceptibility. Diabetes is also characterised by an increase in oxidative stress. Lagman et al.\(^22\) and Tan et al.\(^23\) have shown that type 2 diabetes patients have lower levels of the antioxidant glutathione (GSH) which when restored in vitro enhances mycobacterial killing. Another potential susceptibility factor, namely Vitamin D influences the immune responses to tuberculosis\(^24,25\). Patients with TB, DM or TB-DM all present with reduced levels of Vitamin D\(^26,27\). Moreover, Chaudhary et al. reported that the prevalence of severe vitamin D deficiency was higher in people with TB-DM (45%) as compared with only TB (26.66%), only diabetes (17.39%) or healthy controls (7.69%)\(^26\). This decrease in Vitamin D may contribute to the excess inflammation and disease severity observed in patients with TB-DM.
A concluding picture on the factors influencing TB susceptibility in DM patients

In the first part of this thesis I evaluated the effects of hyperglycaemia on the immune response to *M. tuberculosis*. I found that hyperglycaemia does not have a strong effect in vitro. In vivo patients with T1DM have reduced IL-1B signalling, but this is not correlated with hyperglycaemia. Using butyrate, I have shown that short chain fatty acids can modulate the immune response to *M. tuberculosis* via IL-10.

My work and the published studies described above suggest that there is no single clear cut susceptibility factor. It appears that a multitude of factors converge to create a niche environment in which mycobacteria can evade clearance, reactivate and push the host immune system into over drive (Figure 1). Attempts to understand the precise molecular mechanisms behind each of these factors are ongoing. In the meantime, clinical strategies that reduce the levels of hyperglycaemia, insulin resistance, triglycerides and other factors in TB-DM are recommended. In light of the excess inflammation that characterises TB-DM drugs that limit inflammation should be considered.

Last but not least the ongoing global TANDEM study, under which this thesis falls, will reveal more details on biomarkers, geographic specific differences and the influence of genetics on TB-DM. It is with such concerted effort that we as scientists might have a fighting chance against the potential resurgence in TB.
Figure 1 | Overview of factors with potential to influence the susceptibility of diabetes patients to tuberculosis. It is hypothesized that the immune response of a patient with diabetes to M. tuberculosis is strongly influenced by biological and physiological factors such as dyslipidaemia, insulin resistance, hyperglycaemia and genetics. In the early stages of infection insufficient innate immune responses such as poor recognition, low phagocytic activity, reduced myeloid cell frequencies and a decrease in the release of cytotoxic molecules may prevent early clearance of mycobacteria. Impaired antigen processing and presentation may further delay the immune response. In fact, a study in mice showed that IFN-γ producing T cells appeared later in the thoracic lymph nodes of diabetic mice when compared to control mice. The T-cell profile of patients with diabetes and tuberculosis is also altered. For example in these patients, CD8+ T cells produce increased amounts of pro-inflammatory cytokines but reduced levels of cytotoxic molecules. Granulomas form to wall of infectious or foreign substances that the body cannot eliminate. It is likely that a greater influx of neutrophils and increased production of pro-inflammatory cytokines or factors such as VEGF and matrix metalloproteinases (MMPs) could lead to the formation of a necrotic core. This would promote increased bacterial persistence, growth and eventual dissemination. These factors could converge to form a granuloma that suffers from excessive pathology and poor bacterial control potentially leading to bacterial dissemination.
PART II

Cellular metabolism of immune cells and host defence to tuberculosis
SUMMARY

Mounting an immune response to an invading pathogen requires more energy. Recent technological advances have led to an explosion of studies that seek to understand the metabolic processes that underpin the cellular activation, differentiation and function of immune cells. In particular, the processes of glycolysis and OXPHOS have come under the spotlight and they have been shown to drive host responses to stimulation with TLR ligands \[^{36, 37}\].

In Chapter 5 we characterised the changes in glucose metabolism that take place during \(M.\) \(tuberculosis\) stimulation. We showed that glucose metabolism is increased in the circulating blood cells of patients with active tuberculosis and that it tapers back down to normal after treatment. We replicated this in vitro and demonstrated that the activation of glycolysis took place via the TLR2 – AKT – mTOR axis. Inhibition of any of these axes using pharmacological inhibitors or genetic knockouts led to an in vitro and in vivo decrease in immune responses to tuberculosis thereby confirming the importance of glycolysis in the immune response to \(M.\) \(tuberculosis\).

The Warburg effect, which describes a switch from OXPHOS to glycolysis, has been adopted as a core paradigm in the field of immune metabolism. It primarily describes the rewiring of cellular metabolism in myeloid cells upon stimulation, with LPS as the model stimulus \[^{36, 38}\].

In Chapter 6 we investigated whether this switch also takes place in the context of other stimuli and looked for the functional importance of OXPHOS. To our surprise, we discovered that LPS was unique in downregulating OXPHOS, whilst all other stimuli including the TLR2 ligand Pam3Cys, \(E.\) \(coli\), \(M.\) \(tuberculosis\) and \(S.\) \(aureus\) increased OXPHOS. Further investigation revealed that the dose of LPS influenced cellular OXPHOS rates and that OXPHOS is functionally linked to the process of phagocytosis.

Glycolysis and OXPHOS, however, are just two of many metabolic pathways. Therefore, in Chapter 7 we used a human tissue infection model \[^{39}\] to perform a systems analysis of the most differentially regulated metabolic pathways during \(M.\) \(tuberculosis\) infection. This yielded a 292-gene signature from which the relative importance of each gene was tested using cytokine-QTL analysis. Next, we used this gene set to examine the influence of genetic variation in metabolic genes on cytokine production in response to \(M.\) \(tuberculosis\). It confirmed the importance of several metabolic pathways such as the glycolysis, tryptophan and glutathione pathways which have previously been linked to tuberculosis. It also highlighted several undescribed pathways such as the pyrimidine and inositol-phosphate
metabolic pathways in the context of TB. Through this approach, we have identified several previously unknown metabolic genes that are at the crossroads between cellular metabolism and the immune system, and as such represent relevant therapeutic targets.

An important question that arose from the work above was the therapeutic potential of this data. To answer that we turned to a recent study that put forward metformin, the most widely administered diabetes drug, as an adjunctive therapy for tuberculosis. In Chapter 8 we investigated the immune modulating effects of metformin in a group of healthy volunteers taking metformin for five days. This study revealed a profound change in the transcriptional and cellular landscape of metformin with a specific decrease in pro-inflammatory cytokines and increase in ROS and phagocytosis. Mechanistically, inhibition of the MAPK and AKT axes was observed whereas AMPK and glycolysis were increased. Although no direct influence on killing was seen ex vivo, it is likely that a systemic model encompassing all the above changes is needed to see the anti-mycobacterial effects of metformin. This study supports the possibility of metformin as adjunctive therapy for tuberculosis but also highlights the caution with which this should be done.
DISCUSSION

Based on these chapters the following question arises: **How does cellular metabolism influence individual host defence mechanisms against M. tuberculosis infection?**

Some of the mechanisms employed in response to *M. tuberculosis* include (but are not limited to) cytokine production, ROS formation, phagocytosis, autophagy and antigen presentation. Only a couple of these mechanisms have had the metabolic pathways that control them identified. For instance, *M. tuberculosis* upregulates aerobic glycolysis, a process that controls cytokine production and the clearance of mycobacteria from infected macrophages. Similarly, the activity of enzymes controlling the mitochondrial process of OXPHOS is also increased. In the context of TLR2 stimulation, OXPHOS is important for phagocytosis. As mycobacteria signal strongly through TLR2 we can extrapolate that OXPHOS is likely to control the phagocytosis of exogenous mycobacteria too.

Studies by other groups have shown that tryptophan metabolism in *M. tuberculosis* infected macrophages promotes IL-1β and IL-23 synthesis and increasing glutathione levels can enhance Th1 responses to *M. tuberculosis* infected PBMCs. Our systems analysis using a human model of mycobacterial tissue infection confirmed a role for a number of these pathways and identified the pyrimidine and inositol phosphate pathways as additional contributors to the host response to mycobacteria. Although the functions that these pathways control are currently unknown, pyrimidine metabolism has been associated with regulating inflammasome activity and inositol phosphate metabolism is responsible for generating the backbone of the *M. tuberculosis* related signal transduction components IP3 and AKT.

Last but not least, other studies of immune cells have shown that:

- Glycolysis also supports antigen presentation in dendritic cells
- The TCA cycle facilitates production of immunomodulatory metabolites like citrate (proliferation), itaconate (bactericidal) and succinate (cytokine production).
- The pentose phosphate pathway supports ROS production and
- Amino acid metabolism supports proliferation and T effector cell function.
To conclude

In this thesis, I have shown that glycolysis is important for *M. tuberculosis* induced cytokine responses via the AKT-mTOR pathway. I have then showed that LPS is unique in down-regulating OXPHOS whilst other stimuli upregulate OXPHOS. I showed that OXPHOS plays a role in phagocytosis. Using a systems biology approach, I then identified pyrimidine metabolism and inositol phosphate signalling as novel and important metabolic pathways in the host response to *M. tuberculosis*. Lastly, I have provided evidence supporting the concept that metformin, a diabetes drug, is a candidate for host-directed therapy in tuberculosis.

Future studies should focus on the specificity of these pathways during *M. tuberculosis* infection and should include other TB relevant host defence functions such as autophagy and ROS production. To date, nearly all *M. tuberculosis* related metabolism studies are based on gene expression data which do not account for changes in enzyme activity and substrate availability. Our systems analysis above identified several key genes that controlled amino acid metabolism. Using more advanced techniques, such as metabolomics and flux analyses of infected cells, will help us to identify exactly which amino acids are important during infection. With improved techniques and representative infection models we will be able to investigate how these and other pathways play a role in controlling host defences to *M. tuberculosis*. 
FUTURE PERSPECTIVES
As this thesis comes to a close the final question I ask myself is the following: Can we harness discoveries in the field of immune-metabolism to improve the outcome of TB patients regardless of DM status?

Current tuberculosis treatment time ranges from a standard 9-month regimen anywhere up to 24 months for drug resistant TB, making this one of the longest treatment regimens that exists for any bacterial disease. Failure is still common in part due to side effects, drug toxicity, poor adherence and increasing drug resistance. The lack of new TB drug classes since the 1970s has blunted progress in improving treatment time and tackling drug resistance. Additionally, antibiotics themselves do not deal with the inflammation, tissue damage and paradoxical responses (IRIS) that encompass the host response. These points in fact lay down a strong case for a novel approach to treating tuberculosis and in particular for host directed therapy (HDT) combination therapy where both pathogen and host are targeted.

Recent papers in this thesis and elsewhere have linked host cell metabolism to inflammation and, in the case of M. tuberculosis, bacterial clearance. Metabolism modulating drugs are used to treat many diseases. Thus, never before have we had such a large repository of candidate FDA approved drugs for putative adjunctive TB therapy. Identification of candidates must be done systematically. Firstly, the aim of metabolism-based HDT would be to reduce tissue damage and excess inflammation whilst improving bacterial clearance. Secondly, as researchers we need to improve our knowledge of which host immune functions, important for defence against TB, are controlled metabolically and by which pathways.

Metformin

To begin, metformin stands at the forefront of candidate HDT drugs. It is FDA-approved, widely used among patients with diabetes and increases glycolysis whilst inhibiting mTOR. This results in a decrease in inflammation and increase in mycobactericidal processes such as ROS production, autophagy and phagocytosis. The process of autophagy, a potentially protective mechanism against tuberculosis, is activated by metformin and AICAR, another AMPK activator, and is negatively correlated with mTOR activity. Clinically approved drugs like sirolimus and rapamycin enhance autophagy but also inhibit mTOR and inflammation. However, their potent immune suppressing properties and drug-drug interaction may prevent them from being used as HDT.
Statins

Statins are cholesterol-lowering drugs which in several studies have been shown to reduce the mycobacterial burden in mice. Clinically, familial hypercholesterolemia (FH) patients on atorvastatin controlled *M. tuberculosis* infection better than matched healthy controls. Mechanistically, a reduction in cholesterol via the HMG-CoA –mevalonate pathway was suggested to improve phagosome maturation and autophagy. Nonetheless, evidence also suggests that low cholesterol levels are a risk factor for tuberculosis. Cholesterol-depleted mouse macrophages decrease their ability to phagocytose mycobacteria, whilst the acute phase response in tuberculosis patients is characterised with low serum cholesterol. Additionally, a cholesterol-rich diet during treatment accelerated the rate of sputum sterilisation in new hospitalised cases of pulmonary tuberculosis. An important aspect of HDT is the pharmacokinetics of drugs. Human studies have shown that rifampicin greatly decreases the plasma concentrations of simvastatin and its combination with isoniazid, an anti-tuberculosis drug, may increase the risk of myopathy and rhabdomyolysis. Overall, statins are promising HDT candidates as they reduce high cholesterol levels and improve anti-mycobactericidal activities whilst decreasing cellular inflammation. Their use in tuberculosis treatment may be limited to specific patient groups with high cholesterol levels such as T2DM patients or persons at risk for cardiovascular disease.

Drugs modulating arachidonic acid metabolism

Last but not least arachidonic acid metabolism has been in the spotlight for tuberculosis in recent years. Prostaglandin E2 (PGE2), a metabolite of arachidonic acid, has been shown to limit the excessive anti-inflammatory effects of type I interferon signalling, a core signature of pulmonary TB patients. Therapeutically zileuton, a clinically approved drug for asthma, works by tipping arachidonic acid metabolism in the direction of increased PGE2 production. This protects mice from disease and establishes yet another potential avenue for HDT in tuberculosis.

Covering every mode of potential metabolism-related HDT is the foreground for future research and Figure 2 is an illustration summarising the possible avenues.

The figure highlights several FDA-approved or research drugs at different stages of clinical development that could potentially be repurposed for HDT in tuberculosis by targeting metabolic and / or immune mechanisms. Targeting these pathways for HDT should be high on the agenda of HDT specialists and clinicians looking for improved TB therapeutics.
Finally, despite decades of attempts on eradication, tuberculosis remains a global threat. Using novel but efficient strategies, like HDT, and close monitoring of high risk groups, like patients with diabetes, we as researchers can continue to work towards the goal of full TB elimination by 2050.

Figure 2 | FDA-approved or research drugs with HDT potential for tuberculosis. The figure highlights several drugs in different stages of clinical development with potential for HDT in tuberculosis. The drugs mainly target metabolic and/or immune modulatory pathways. Some of these include inflammation-related pathways, tryptophan metabolism, cellular energy metabolism pathways mediated by mTOR or AMPK, NAD+/NADH metabolism and cholesterol metabolism. Exploring these pathways for their HDT potential should be high on the agenda of HDT specialists and clinicians looking for improved TB therapeutics.
REFERENCES


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Chapter 10

Nederlandse Samenvatting
TUBERCULOSE

Tuberculose (TB) is een infectieziekte, die veroorzaakt wordt door de bacterie *Mycobacterium tuberculosis* (*M. tuberculosis*), en heeft zich ontwikkeld tot een zeer moeilijk uitroeibare ziekte. In 2015 alleen al zijn er 1,8 miljoen mensen overleden aan TB en kregen er 10,4 miljoen mensen de ziekte TB. Volgens de WHO komt 60% van alle ziektegevallen voor in slechts zes landen: India, Indonesië, China, Nigeria, Pakistan en Zuid-Afrika.

Deel 1: Tuberculose en Diabetes

Het is bekend dat het hebben van diabetes in 15% van de tuberculose gevallen een rol speelt. In 2015 waren er 415 miljoen mensen met diabetes, en de verwachting is dat dit zal oplopen naar 642 miljoen in 2040. Omdat diabetes een rol speelt in tuberculose, heeft deze stijging in diabetes ook invloed op het aantal TB gevallen, en is het belangrijk om te weten hoe dit werkt, wat de mechanismen zijn en hoe we dit kunnen voorkomen. Dit is wat ik in het eerste gedeelte van mijn thesis beschrijf.

In hoofdstuk 2 heb ik gekeken naar de effecten van hoge glucose waarden (hyperglycemie) op de immuun respons tegen *M. tuberculosis*. Ik beschrijf dat hyperglycemie niets verandert aan de immuun reactie tegen *M. tuberculosis*, want er is geen verschil op het gebied van cytokine productie en in de capaciteit om *M. tuberculosis* te doden (bacteriële klaring). In hoofdstuk 3 beschrijf ik dat patiënten met type 1 diabetes een verminderde interleukine (IL) 1 beta productie hebben, alhoewel dit niet toe te schrijven is aan hyperglycemie. Tot slot heb ik in hoofdstuk 4 laten zien dat butyraat, een zogenoemd korte keten vetzuur, en andere vergelijkbare metabolieten in de darmen een sterke invloed kunnen hebben op de immuun respons tegen *M. tuberculosis* via IL-10.


**Deel 2: Tuberculose en metabolisme**

De immuun respons tegen een indringend pathogeen kost veel energie. Recente technologische ontwikkelingen hebben geleid tot een explosie aan studies naar de cellulaire processen die zorgen voor deze energie. Deze metabole processen ondersteunen het activeren, de ontwikkeling en de functie van immuuncellen. Twee processen hebben hierbij de spotlight gehaald: de zogenaamde glycolyse en oxidatieve fosforylering (OXPHOS). Zowel de glycolyse als OXPHOS blijken een belangrijke rol te spelen in onze immuun respons, zoals cytokine productie in reactie op de stimulatie met microbiële antigenen (genaamd TLR liganden). In het tweede deel van mijn thesis heb ik de veranderingen in intracellulair metabolisme bestudeerd, en specifiek als gevolg van tuberculose infectie.

In **hoofdstuk 5** heb ik cellen bestudeerd van zowel gezonde mensen als tuberculose patiënten. Hier laat ik zien dat het proces van glycolyse belangrijk is voor de cytokine productie na *M. tuberculosis* infectie. Ook laat ik zien dat deze processen afhankelijk zijn van de AKT-mTOR route. In **hoofdstuk 6** laat ik vervolgens zien dat LPS, een TLR4 ligand, als enige TLR ligand de OXPHOS platlegt. Vele andere TLR liganden, net zoals bijvoorbeeld *M. tuberculosis*, activeren namelijk juist de OXPHOS. Ik laat zien dat de OXPHOS specifiek een rol speelt in de immuun respons waar fagocytose nodig is, om bijvoorbeeld bacteriën te klaren. In **hoofdstuk 7** maak ik gebruik van systeembiologie en hiermee heb ik twee routes geïdentificeerd die belangrijk zijn in de immuunreactie tegen *M. tuberculosis*. Metabolisme van pyrimidine en de inositol fosfaat route blijken nieuwe en zelfs genetisch gezien belangrijke routes te zijn in de immuunreactie tegen *M. tuberculosis*.

Deze studies leiden samen tot het laatste hoofdstuk van mijn thesis, waarin ik deze metabole routes bespreek als nieuwe potentiële therapeutieën tegen TB. In **hoofdstuk 8** heb ik een klinische studie beschreven, waaruit blijkt dat metformine, een veelgebruikt medicijn tegen diabetes, een goede kandidaat is om metabole routes te beïnvloeden en zo eventueel tuberculose tegen te gaan.
Nederlandse Samenvatting

TOEKOMSTPERSPECTIEF

De huidige behandeling van tuberculose varieert van een 9 maanden lange standaardbehandeling tot 24 maanden behandeling voor resistente TB. Dit maakt de behandeling van TB een van de langste behandelplannen van alle bacteriële infecties. Helaas werkt de behandeling niet altijd even goed. Er zijn bijwerkingen, medicatie intoxicatie, slechte naleving van instructies en een groeiende resistentie van TB tegen medicijnen. Sinds 1970 zijn er eigenlijk geen goede nieuwe medicijnen tegen TB meer ontwikkeld. Men is niet in staat de behandeltijd of resistentie te verbeteren. Dit betekent dat er veel ruimte (en nood) is voor een nieuwe benadering van TB behandeling, bijvoorbeeld een behandeling die zowel de pathogene als de patiënt beïnvloedt.

Recente studies in deze thesis maar ook elders hebben een link gelegd tussen het cellulair metabolisme van de patiënt en de bijbehorende ontsteking en - in het geval van M. tuberculosis - bacteriële klaring. Medicijnen die het metabolisme beïnvloeden worden echter al lang gebruikt in de behandeling van vele andere ziekten. Daarom lijkt het alsof we nog nooit eerder zoveel nieuwe kandidaat medicijnen hebben gehad - die overigens al door de FDA zijn goedgekeurd - om tuberculose te bestrijden. De identificatie van goede kandidaten moet systematisch gebeuren. Het eerste doel van behandeling, wat het metabolisme als target heeft, moet de schade aan de weefsels en ontsteking verminderen, maar ook bacteriën opruimen. Ten tweede is het belangrijk dat wij als onderzoekers meer te weten komen over de immuun respons die belangrijk is voor TB, hoe deze metabool worden gecontroleerd en via welke routes.

Er zijn momenteel drie kandidaten voor een behandeling die zowel op de patiënt als de pathogene aangrijpen, gebaseerd op het veranderen van het metabolisme. Metformine, welke ook onderzocht is in deze thesis, staat voorop. Het is een FDA-goedgekeurd medicijn wat al veel gebruikt wordt in diabetes behandeling. Metformine verhoogt de glycolyse, maar remt mTOR. Dit resulteert in een vermindering van ontsteking en een verhoging van de processen die nodig zijn voor mycobacteriële klaring, zoals ROS productie, autofagie en fagocytose. Een tweede kanshebber is statine. Statines zijn cholesterol-verlagende medicijnen die, zoals meerdere studies hebben laten zien, invloed hebben op mycobacteriële infectie in muizen. Ze verlagen hoge cholesterolwaarden maar vergroten ook de anti-mycobacteriële processen en verlagen ontsteking. Deze medicijnen worden tot nu toe alleen gebruikt in tuberculose behandeling als de patiënt ook last heeft van hoog cholesterol, zoals veel type 2 diabetes patiënten of patiëntten met een verhoogd risico op cardiovasculaire ziekten. Hier is dus nog ruimte voor nieuw onderzoek. Ten derde zijn er nieuwe medicijnen die recentelijk meer aandacht hebben gekregen. Deze medicijnen moduleren het metabolisme van arachidonzuur, een aminozuur. Zileuton is een van deze medicijnen...
medicijnen en is al klinisch bewezen effectief voor de behandeling van asthama. Zileuton verandert de PGE2 productie, wat uiteindelijk muizen beschermt tegen ziekte. Het wordt een uitdaging om alle metabolisme-gerelateerde routes te behandelen, maar het is een belangrijk onderwerp voor toekomstig onderzoek. In figuur 2 (hoofdstuk 9) geef ik een illustratie van alle mogelijke behandelroutes.

Ondanks jaren van strijd tegen tuberculose blijft het een wereldwijde bedreiging. Nieuwe en efficiëntere strategieën, zoals bijvoorbeeld het behandelen van zowel patiënt als pathogeen, en het nauw monitoren van risicogroepen zoals patiënten met diabetes, zijn nodig om als onderzoekers samen TB te hebben bestreden in 2050.

Translation by Siroon Bekkering.
Chapter 11

Epilogue: Acknowledgements, List of Publications & Curriculum Vitae
ACKNOWLEDGEMENTS

And so here I am thinking of the best way to start this section. And as I struggle to get the words out of my mouth I realise what a journey it’s been. Not just academically but personally too. I’ve come to meet so many extraordinary people and I have a lifetime of memories to cherish. Also I can’t wait to start a sentence saying “Well when I did my PhD…”. But before that there are some of you that I’d like to sincerely thank.

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Room # 3.02, The B-Hoek, Marije, from hot summers through cold winters we’ve survived each other 😂. The more I’ve gotten to know you over time the more I appreciate your attitude to work and life. I wish you all the best. James, it was fun having someone on the
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this book may take six months to get to you and you’ll probably have to watch a recording of this day none of it would be possible without your love and support. Thank you a million times over! And last but never least, Chris, now you can finally say “I survived my wife’s PhD” :P.
LIST OF PUBLICATIONS


CURRICULUM VITAE

Ekta Lachmandas was born in Bangalore, India on June 11th, 1991. When three months old, she moved to St. Maarten in the Dutch Caribbean. She attended St. Dominic High School and completed her CXC’s in June 2008, majoring in biology, chemistry and physics. In August 2008 she moved to Nijmegen, Netherlands to start a Bachelor’s Degree in Life Sciences, majoring in biochemistry. She completed her preparatory year in 2009 (cum laude).

She moved to London, UK in January 2011 for a six-month internship at University College London. She then moved to Oxford, UK in September 2011 to work on her undergraduate thesis at the Jenner Institute at Oxford University. She received her Bachelor’s Degree in August 2012 (cum laude). In October 2012 she started her PhD project at the department of internal medicine under the mentorship of Prof. dr. Mihai Netea and Prof. dr. Reinout van Crevel.

Ekta presented her results at several international conferences and was awarded a Future of Science Scholarship at a Keystone Conference in Santa Fe, USA. She collaborated closely with other research teams in the Netherlands, United Kingdom, Germany, Sweden and Singapore. As a visiting scientist she spent three months at the A*STAR Institute in Singapore expanding on data from her clinical study. Ekta married Christopher Foster in 2017 and moved to Switzerland.
INVITATION
To attend the public defence of my dissertation:

HOST RESPONSE TO TUBERCULOSIS: IMPACT OF DIABETES & CELLULAR METABOLISM OF IMMUNE CELLS

on Wednesday 15 November 2017 at 14:30
in the Radboud University Aula, Comeniuslaan 2, 6525EP Nijmegen, Netherlands

Reception Borrel & Buff et 18:30 at De Blonde Pater, Houtstraat 62, Nijmegen

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Ekta Lachmandas