Investigating the role of immune-endocrine alterations during type 2 diabetes associated with changes in *Mycobacterium tuberculosis* growth

by

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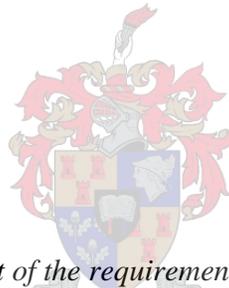
Thesis presented in partial fulfilment of the requirements for the degree of Master of Science (Molecular Biology) in the Faculty of Medicine and Health Sciences at Stellenbosch University

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Declaration

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Abstract

Background

There is a high co-prevalence between type 2 diabetes (DM2) and other infectious diseases. This is particularly problematic with the rise in co-prevalence between DM2 and Tuberculosis (TB). However, the underlying association between TB and DM2 is still poorly understood. We hypothesize that immune-endocrine alterations in latently infected individuals with DM2 are associated with reduced *Mycobacterium tuberculosis* (Mtb)-killing efficacy. We aimed to determine whether Mtb phagocytosis and/or killing efficacy of peripheral blood mononuclear cells (PBMCs) and monocytes (MNs) from close contacts (CCs) of TB patients with or without DM2 is altered and its association with physiological changes characteristic of DM2. In addition, we aimed to identify immune modulatory properties of endogenous hormones such as cortisol, leptin and insulin on PBMCs and MNs of latently infected individuals.

Materials and methods

First, we compared the bacterial burden in PBMCs and MNs of TB close contacts with normal to poorly controlled glycemia during an Mtb infection. We investigated the association between glycemic control, cells counts and hormone signatures, with bacterial burden in DM2 patients. Secondly, we treated PBMCs and MNs with cortisol, leptin and insulin to determine whether these hormones influenced bacterial burden, mycobacterium induced cytokine production and phenotypes of CD4$^+$ and CD8$^+$ T cells.

Results

Bacterial burden was increased in DM2 patients when compared to healthy participants in both PBMCs and MNs. High bacterial burden was associated poor glycemic control. DM2 patients had higher levels of neutrophil counts, white cell counts (WCC) and lymphocyte counts, but low percentage MNs in whole blood compared to healthy participants. Cortisol levels remained unchanged between the groups, however, there was a negative correlation between cortisol and interferon-γ (IFN-γ) (p=0.03, r=0.52) in the DM2 group.

Cortisol, leptin and insulin did not influence the bacterial burden in both PBMCs and MNs. However, the hormones influenced the Mtb induced cytokine production in PBMCs. Cortisol decreased the production of interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α, TNF-β, IL-8, IFN-γ and granulocyte-macrophage colony stimulating factor (GM-CSF). Leptin decreased the production of IL-1RA, IL-13, IL-5, fibroblast growth factor (FGF)-2. Insulin decreased the

production of vascular endothelial growth factor (VEGF), IL-1RA and increased the production of IL-5.

Conclusion

In DM2, phagocytosis and killing efficacy of PBMCs and MNs from CC of TB patients were associated with physiological changes characteristic to DM2. Poor bacterial control in DM2 was associated with hyperglycemia, chronic inflammation induced by increased WCC, neutrophil, and lymphocyte counts. The level of cortisol in DM2 individuals negatively correlated with IFN-γ, thus suppressing Th1 response. Furthermore, the study indicate that endogenous hormones such as cortisol, leptin and insulin could potentially mediate some cytokine response in DM2 patients. Cortisol potentially suppress macrophage activation or Th1 activity, which could lead to poor bacterial control. Whereas, leptin upregulates Th1 response that may improve bacterial control. However, its role still remains undetermined. The role of insulin is debatable as it may either induce a Th2 response, which could lead to poor bacterial control, or may a play a role in preventing the spread of Mtb to other organs by decreasing the production of VEGF. Therefore, during DM2, immune alterations and hyperglycemia are associated with decreased bacterial control, and that endocrine factors such as cortisol would suppress Th1 response, regardless whether it is elevated or not. Thus, would potentially exacerbate bacterial control in cases where DM2 is worsened by other complications.

Opsomming

Agtergrond

Daar is 'n hoë mede-voorkoms tussen tipe 2-diabetes (DM2) en met ander aansteeklike siektes, wat op sy beurt baie druk op gesondheidsorg plaas, in gemeenskappe met beperkte hulpbronne. Dit is veral 'n probleem met die styging in mede-voorkoms tussen DM2 en Tuberkulose (TB). Maar die onderliggende verband tussen TB en DM2 is steeds onduidelik. Ons hipotese stel voor dat immuun-endokriene afwykings in latente infekte individue met DM2 verband hou met verminderde doodmaak doeltreffendheid van *Mycobacterium tuberculosis* (Mtb). Ons het gepoog om vas te stel of Mtb fagositose en of uitwissing geaffekteer word in perifere bloed mononukleêre selle (PBMCs) en monosiete (MNS) van nabye kontakte van TB-pasiënte (CC) met of sonder DM2 verander en die verbintenis met fisiologiese veranderinge, kenmerkend aan DM2, te bepaal. Verder bepaal ons watter immuun-regulerende eienskappe endogene hormone soos kortisol, leptien en insulien op PBMCs en MNS tydens Mtb infeksie het.

Metodes

Eerstens, vergelyk ons die bakteriële las in PBMCs en MNS van CCs met normale en hoë bloedsuiker vlakke. Ons ondersoek die verband tussen glukemiese beheer, sel tellings en hormoon patrone, met bakteriële las in die selle van DM2 pasiënte. Tweedens, het ons PBMCs en MNS met kortisol, leptien en insulien behandel om te bepaal hoe hierdie hormone bakteriële las beïnvloed, sitokien produksie na Mtb infeksies asook fenotipe van CD4$^+$ and CD8$^+$ T sells.

Resultate

Bakteriële las is verhoog in DM2 pasiënte in vergelyking met gesonde deelnemers in beide PBMCs en MNS. Hoë bakteriële las hou verband met swak glukemiese beheer. DM2 pasiënte het hoër neutrofiele, wit sel en limfosiet tellings maar 'n laer persentasie MNS in bloed in vergelyking met gesonde deelnemers. Kortisol vlakke het onveranderd gebly tussen die groepe, terwyl daar 'n negatiewe korrelasie tussen kortisol en interferon-γ (IFN-γ) was (p = 0,03, r = 0.52). Kortisol, leptien en insulien het geen invloed op die bakteriële las in beide PBMCs en MNS.,maar die hormone beïnvloed die sitokien produksie van PBMCs wat met Mtb geinfekteer is. Kortisol

inhibeer die produksie van interlukin (IL) -1β, IL-6, tumer nekrose faktor (TNF) -α, TNF-β, IL-8, IFN-γ en granulocyte-makrofaag kolonie stimulerende faktor (GM-CSF). Leptien inhibeer die produksie van IL-1RA, IL-13, IL-5, fibroblast groeifaktor (FGF) -2. Insulien inhibeer die produksie van vaskulêre endoteel groeifaktor (VEGF) en IL-1RA en stimuleer die produksie van IL-5.

Afsluiting

Swak bakteriële beheer in DM2 is toegeskryf aan hoë bloedsuiker vlakke, chroniese inflammasie veroorsaak deur 'n toename in wit bloed, neutrofiel en limfosiet sel tellings. . Die vlak van kortisol in DM2 individue was negatief gekorreleer met IFN-γ, dus kon potensieel Th1 reaksie onderdruk. Endogene hormone soos kortisol, leptien en insulien kan sommige cytokine-reaksie by DM2 pasiënte bemiddel. Kortisol verminder óf makrofage aktivering of Th1 aktiwiteit, wat kan lei tot swak bakteriële beheer. Leptien aan die ander kant induseer 'n Th1 reaksie wat bakteriële beheer kan verbeter. Die rol van insulien is onduidelik, aangesien dit ook 'n Th2 reaksie kan induseer, wat kan lei tot swak bakteriële beheer, of dalk 'n 'n rol speel in die voorkoming van die verspreiding van Mtb na ander organe deur die produksie van VGEF te verminder. Tydens DM2 is immuun veranderings en hoë bloedsuiker vlakke geassosieer met verlaagde bakteriële beheer, en dat endokriene faktore soos kortisol sou Th1 reaksie onderdruk, ongeag of dit verhoog is of nie. Dus, sou potensieel vererger bakteriële beheer in gevalle waar DM2 is vererger word deur ander komplikasies.

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List of abbreviations

11-HSD1	11β-hydroxysteroid dehydrogenase type 1
ACTH	Adrenocorticotropin
AGEs	advanced glycation end products
APC	antigen-presenting cell
BMI	body mass index
BSA	bovine serum albumin
CC	close contacts
CFUs	colony forming units
CNS	central nervous system
CRH	corticotropin-releasing hormone
DHEA	Dehydroepiandrosterone
DM	Diabetes
DM2	Type 2 diabetes
DMSO	dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FBG	fasting blood glucose
FBS	fetal bovine serum
FGF	fibroblast growth factor
GC	Glucocorticoids
Glu	Glutamine
GM-CSF	Granulocyte macrophage colony-stimulating factor
Hb	Haemoglobin
HC	Healthy controls with no DM
HH	Healthy controls
HHC	household contacts
Hi	heat inactivated
HIV	Human Immunodeficiency Virus
HPA	hypothalamus-pituitary-adrenocortical axis
HREC	Human Research Ethics Committee
hsCRP	highly sensitivity C-reactive protein

IFN-	interferon-
IGRA	Interferon-Gamma Release Assays
IL-	interleukin-
IP-	interferon gamma-induced protein
IR	insulin resistance
LPS	Lipopolysaccharide
LTBI	latent TB infection
MCP-	monocyte chemoattractant protein
MC-SF	macrophage colony-stimulating factor
MDM	monocyte derived macrophages
MGIT	Mycobacteria growth indicator tube
MN	Monocytes
MOI	multiplicty of infection
Mtb	Mycobacterium tuberculosis
NK cells	natural killer cells
NOS	nitrogen oxidative species
OADC	Oleic Albumin Dextrose Catalase Growth Supplement
PBMC	peripherial blood mononuclear cells
pDM2	poorly controlled diabetes
Pen	Penicillin
PHA	phytohaemagglutinin
preDM2	pre-diabetes
ROS	reactive oxidative species
RT	room temperature
SST	serum-separating tubes
TB	Tuberculosis
TLR-	toll like receptor
TNF-	tumor necrosis factor-
VEGF	vascular endothelial growth factor
WCC	white cell count

Chapter 1

Introduction

1. Diabetes and Tuberculosis

1.1. Type 2 diabetes

Type 2 diabetes (DM2), also known as adult-onset diabetes mellitus, is a condition that often develops in overweight middle-aged and older adults (aged between 45 to 64), but has also been diagnosed in children, teens and young adults (American Diabetes Association 2010; Hillier and Pedula 2003). The etiology of the disease is when uncontrolled, high blood glucose levels as a result of either a lack of insulin or ineffective use of insulin by the body occurs (Nicolau et al. 2016). This is also referred to as insulin resistance. This is led by chronic, low-grade inflammation and oxidative stress, which in turn cause pancreatic islet β-cell dysfunction, which would lead to insulin resistance in skeletal muscle, liver and adipose tissue (Hodgson et al. 2015). Type 1 diabetes, on the other hand, generally develops at a young age and results in autoimmune destruction of pancreatic islet β-cell (Daneman 2006). Rendering the pancreas to produce either little or no insulin (Daneman 2006).

The severity of DM2 vary amongst individuals: some people manage their condition by either making a few lifestyle changes such as changing to a low carbohydrate-high fiber diet, exercising or even bariatric surgery to lose weight, others need more permanent therapy that involves taking medication (acarbose, miglitol and metaformin etc.) or even insulin injections (Taylor 2013).

Many people are not always aware that they have diabetes, but may experience symptoms such as continuous thirst, frequent urination, fatigue, nausea and dizziness (Schlienger 2013). In more severe cases, the following could occur; high blood pressure resulting in macrovascular complications (which could lead to amputations) or microvascular complications because of high glucose levels which includes retinopathy, neuropathy and nephropathy (damage to the retina, nerves and kidneys respectively) (Schlienger 2013). Other complications include proteinuria and hyperinsulinaemia (high insulin levels in the blood) and diabetic ketoacidosis (high levels of ketones, because of the breakdown of fat due to insufficient insulin). In some cases when extremely high levels of glucose circulate the blood, individuals could go into diabetic coma (Schlienger 2013).

When diagnosing diabetes, glucose levels are measured in blood. One of the most common methods used to test for diabetes includes testing for Haemoglobin A1c (HbA1c) in the blood (American Diabetes Association 2010). In principle, glucose bind to haemoglobin (Hb) on red blood cells resulting in "glycosylated Hb". Therefore, the more glucose circulating, higher the percentage of glycosylated HbA1c there would be. This is an effective means of diagnosing diabetes, since red blood cells have an average life cycle of 8-12 weeks, thus indicating how high blood glucose levels have been over 8-12-week period therefore not influenced by recent sugar intake. The level of HbA1c is used to assess the "diabetic state" of the individual: normal non-diabetes <5.7%, pre-diabetes (preDM2) 5.7-6.4%, type 2 diabetes (DM2) 6.5-7.9% and poorly controlled diabetes (pDM2) ≥8% (American Diabetes Association 2010). Other blood tests include glucose tests, which either test the amount of glucose in fasting or non-fasting individuals, how quickly the glucose is removed in the blood and how much glucose is in the blood 2 hours after eating. In addition, doctors use urine test to determine whether diabetes is managed properly by monitoring the glucose and ketones in the urine (American Diabetes Association 2010).

Diabetes is a growing epidemic, which affected 382 million people in 2013 (IDF 2014), and this number is predicted to increase to 592 million people by the year 2035 (Guariguata et al. 2014). The global prevalence of DM is attributed to DM2 in about 85-95% of individuals. Currently, more than 80% of people with DM2 live in low and middle-income countries (Beagley et al. 2014). This is particularly problematic as it puts a lot of strain on healthcare systems in these resource-limited regions. DM2 on its own is a difficult disease to control and with the addition of high co-prevalence of infectious diseases, DM2 patients are more likely to visit primary healthcare facilities compared to those without DM2 (Hine et al. 2016). There is a strong positive association between DM2 and infectious diseases including but not limited to genital and perineal, skin and soft tissue and urinary tract infections (Hine et al. 2016). Furthermore, diabetes is associated with Tuberculosis (TB) at a relative risk of 1.2 to 7.8, with the lowest estimates reported in low-TB burden countries (Dooley and Chaisson 2009). It is estimated that 15% of the worldwide TB-burden is attributed to DM2. In South Africa, particularly in the Western Cape, the coloured community has the second highest prevalence of DM2 (28%) (Erasmus et al. 2012), and a high TB prevalence of (24/1000) (Claassens et al. 2013).

1.2. Tuberculosis

There were approximately 9.6 million active TB incident cases and accounted for 1.5 million deaths in 2014 globally. The South African TB incident rate is > 250-fold higher (834 cases per 100 000 population annually) compared to the United States which only has 3 cases per 100 000 population annually (Figure 1).
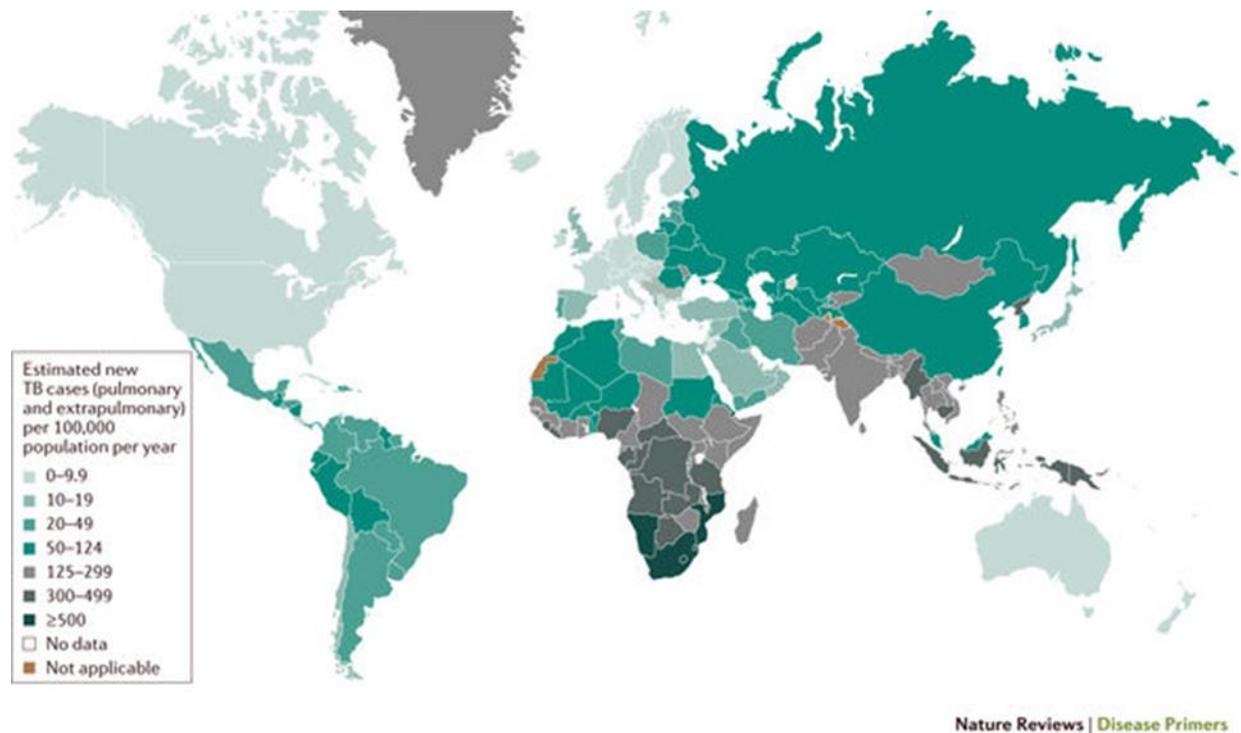
Figure 1. Global active TB incidence rate. Low active TB incident rates (< 10 cases per 100,000 annually) are situated in developed countries such as Canada, Australia, Europe, New Zealand and the United States. By contrast, high active TB incident rates are situated in undeveloped countries especially those situated in Africa.

Taken from (Pai et al. 2016)

Mycobacterium tuberculosis (Mtb) causes TB (Kaufmann 2006). The immune response against Mtb infection is mediated by the early reaction of the innate immune system, which is primarily focused on the phagocytosis, killing or controlling the growth of the pathogen by means of activated macrophages and other innate immune cells including neutrophils and dendritic cells (Kaufmann 2006). This initial cellular immune response is supported by antigen-specific T cells

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producing pro-inflammatory cytokines such as interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α) and interleukin-12 (IL-12) (A. M. Cooper 2009). As the disease progresses the protective cellular immune response is gradually lost and when the pathogen cannot be controlled by the initial immune response a systemic response follows (Mahuad et al. 2004; Natalia Santucci et al. 2011a). This response is characterized by neuroendocrine and metabolic changes that leads to changes in homeostasis that support host defense (Natalia Santucci et al. 2011a). The nutritional status, energy expenditure and hormonal signals of an organism are regulated in such a way to favour a protective immune response by influencing the microenvironment in which the immune cells exert their function. This refers to factors such as BMI and regulation of hormones such cortisol and leptin amongst others (Natalia Santucci et al. 2011a).

Given the complexity of the etiology of DM2 and its many associated complications, the mechanism underlying the association between diabetes and bacterial infections such as Mtb is poorly understood. Herein, we would further explore possible hypotheses including poor glycemic control and a dysregulated endocrine system which could contribute to the increased susceptibly to Mtb.

2. DM2 associated hyperglycemia and TB susceptibility

Glycemic control is a term used to refer to levels of blood sugar (glucose) in a person with DM. Individuals with DM2 is tasked with maintaining euglycemic blood glucose levels, defined as plasma glucose approximately 60 mg/dl (Perlmuter et al. 2008). DM patients with high HbA1c are considered to have poor glucose control. Patients with carefully controlled blood glucose are no more susceptible to bacterial infections than healthy individuals (Gan 2013), while bacterial infections were commonly found DM2 individuals with poor glycemic control, particularly hyperglycemia (Hine et al. 2016).

Since a major aspect of DM2 is hyperglycemia, this would suggest that hyperglycemia would influence immune response in DM2 individuals. Work done by Lecube *et al.* showed attenuated phagocytosis of peripheral blood mononuclear cells (PBMCs) in patients with DM2 correlated with poor glycemic control, and this could be explained by lower activation of macrophages (Lecube et al. 2011). They further showed that this can reversed when improving blood glucose levels (Lecube et al. 2011). A defect in phagocytic function would lead to difficulty to clear bacterial function. Treating monocyte derived macrophages (MDMs) with glucose (16-22mM) reduced

phagocytosis of Mtb (Montoya-Rosales et al. 2016). Monocytes (MNs) of DM2 patients have a reduced phagocytic and antibacterial activity against Mtb (Restrepo et al. 2014).

Several kinds of molecules such as opsonins and complement factors in the blood and extracellular fluid bind to microbes and promote innate response during an infection (Sherwood and Toliver-Kinsky 2004). To induce a proper immune response, there should be effective binding between the pathogen and innate immune cells such as macrophages, neutrophils and dendritic cells to phagocytose the pathogen (Sherwood and Toliver-Kinsky 2004). Gomez *et al.* showed that MNs from patients with DM2 who are Mtb-naïve have reduced association (attachment and phagocytosis) with Mtb compared to non-DM2 individuals (Gomez et al. 2013). This would suggest that host cell recognition is altered, which could potentially lead to alterations in the intracellular fate of the bacterium. Based on further experiments with Mtb opsonized with heat-inactivated serum led them to speculate that DM2 patients have alterations in complement factors which could potentially impair Mtb binding to MNs (Gomez et al. 2013). This led to question whether the reduced association between the microbe and MN was a result of either a defect in the expression or function of major receptors. The same group, however, did not find any changes in cell surface expression of FcRs or CR3 on DM2 MNs to explain the reduced phagocytic capacity via the FcR or CRs. It was therefore speculated that there was a functional defect in CR- and FcR-mediated phagocytosis (Restrepo et al. 2014), and alterations in serum complement was not ruled out.

After binding of the pathogen to the immune cell, there are soluble mediators such as cytokines and chemokines secreted by innate immune cells that promote an inflammatory response (Sherwood and Toliver-Kinsky 2004). Once an immune response is induced, more phagocytes or other immune cells are recruited to the site of infection and they may be directly involved in the killing of microbes (Sherwood and Toliver-Kinsky 2004). During an Mtb infection, macrophages produce IL-12, which contributes to cell-mediated immune response by promoting differentiation of naïve T cells and IFN-γ production that is vital in immunity against Mtb (Robinson and Nau 2008). IFN-γ is essential in the activation of macrophages, allowing them to exert its microbicidal roles (Robinson and Nau 2008). Tan *et al.* reasoned impaired phagocytosis in DM2 PBMCs were due to decreased IL-12 production which in turn led to a decrease in IFN-γ production (Tan et al. 2012). They further showed in PBMCs that low IL-12 production in response to Mtb is toll like receptor (TLR)-2, -4 and 5 independent (Tan et al. 2012). When whole blood was stimulated with Mtb, IL-12 production was not reduced in DM2 patients (Stalenhoef et al. 2008). The variation in IL-12 production could be attributed to the lack of control of the number of cells in a whole blood

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assay, thus influencing the number of cytokine producing cells (e.g. the number of monocytes to produce IL-12). However, others suggest that hyperglycemia does not influence the phagocytic capacity of macrophages or the control of Mtb growth but rather cytokine secretion. In one of these studies, Lachmandas *et al* showed that hyperglycemia altered cytokine secretion of macrophages during Mtb infection by increasing IL-6, IL-10 and IL-1RA and TNF-α production (Lachmandas et al. 2015).

In mice, chronic hyperglycemia contributes to a temporary delay, but otherwise seemingly unimpaired cellular immune response to Mtb, which resulted in a higher plateau of the bacillary burden (Martens et al. 2007). Ultimately resulting in more extensive inflammation. This would suggest that these delays would likely contribute to a higher risk for DM2 patients to develop TB, since efficient phagocytosis and priming of the adaptive immune responses are essential to induce a cell-mediated immune response to hamper the initial Mtb growth.

Apart from the innate immune response, the adaptive immune response was also implicated in the susceptibility of DM2 patients to TB. This was associated with the defective Th1 cytokine response observed in DM2 patients. In individuals latently infected with Mtb, reduced levels of the Th1 cytokines IFN-γ, TNF-α and IL-2 together with reduced levels of IL-17 was associated with pre-DM2 or DM2 status (Kumar, George, et al. 2014).

Discrepancies in studies could be due to differences in the study designs and associated factors such as age, HbA1c levels, metabolic distresses and medication used. Nevertheless, DM2 is a much more complicated disease to be merely explained by hyperglycemia. We hypothesize that a possible reason for the increased susceptibility to Mtb is a combination of hyperglycemia and a dysregulation of the endocrine system during diabetes. As with all complex organisms, single biological systems rarely work in isolation. There is extensive cross-talk between the immune and endocrine axis, together with the neural system, to form the major communication network in the body, and this can have implications in immune surveillance.

3. Involvement of the HPA axis in DM2 and TB

Metabolic alterations have been linked to the increase activity of the hypothalamus-pituitary-adrenocortical (HPA) axis in DM2. We therefore have to consider the contribution of the HPA axis to the susceptibility to infectious diseases in DM2. The HPA axis is a tightly regulated system, which is activated in response to acute and chronic physiological stress (Diz-Chaves et al. 2016).

This is in response to stimuli sent by the endocrine, nervous and immune system that activates the hypothalamus, which in turn result in the release of corticotropin-releasing hormone (CRH) (Diz-Chaves et al. 2016; Glaser and Kiecolt-Glaser 2005) (Figure 2). CRH stimulates the anterior pituitary gland to release adrenocorticotropin (ACTH) that in return stimulates the release of glucocorticoids such as cortisol from the adrenal glands (Figure 2). This results in a cascade of physiological events. Once the stressor has resolved, the response is terminated through a negative feedback loop. However in DM2 it has been debated that the activity of the HPA is enhanced (Stephens et al. 2016; Steptoe et al. 2014). This is evident through the elevated urinary-free cortisol and increased ACTH-induced cortisol levels. This could potentially have downstream effects, which influence how the body respond to infections.
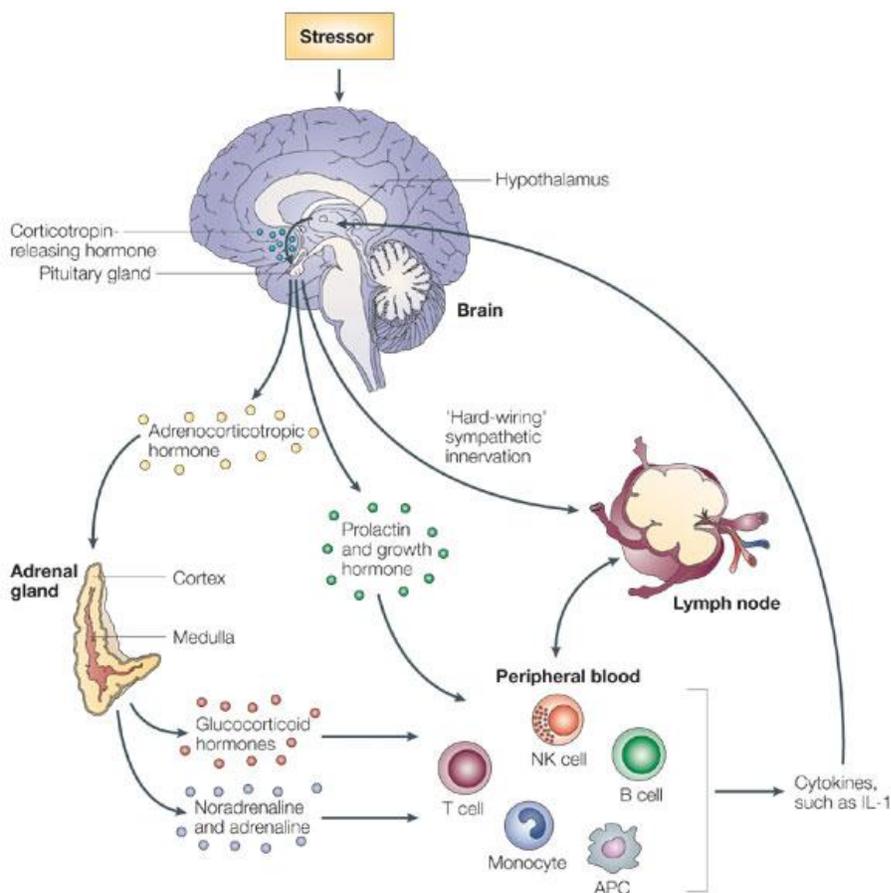
Figure 1. Stress-associated modulation of hormone response by central nervous system.
Abrv. APC, antigen-presenting cell; IL-1, interleukin-1; NK, natural killer.
Taken from (Glaser and Kiecolt-Glaser 2005)

3.1.   Cortisol: A link between DM2 and TB?

Cortisol a steroid-based hormone, $C_{21}H_{30}O_5$, is synthesized from cholesterol and belongs to a group of hormones called glucocorticoids (GC). The level of cortisol in the body is dependent on the type of stress the body is experiencing (Stephens et al. 2016). Under normal conditions, cortisol follow a strong circadian rhythm across the day, upon awakening the hormone is elevated, and gradually decline over the remainder of the day, reaching a nadir 18+ hour after awakening (Rohatagi et al. 1996). Cortisol is a major regulator of energy metabolism; inducing gluconeogenesis, lipogenesis and inhibiting free fatty acid oxidation in the liver, inhibiting glucose uptake and glycogen synthesis in the muscle and in the pancreas and impairing glucose uptake and oxidation (Straub and Cutolo 2016). Apart from metabolic functions, cortisol is known to influence the immune system.

The level of cortisol varies in patients with DM2 as some studies have found that these patients have higher basal cortisol levels, increased plasma cortisol after dexamethasone suppression test, higher 24 h urine-free cortisol and increased gland volume when compared to individuals without DM2 (Bruehl et al. 2007). However, other studies suggest that this increase in cortisol is dependent on the severity of DM2 based on the complications accompanying with the disease (Chiodini et al. 2007). Recently, a longitudinal study investigated the association between DM2 and long-term changes in daily cortisol curve features and did not find any differences in the change in cortisol trends by diabetes status (Spanakis et al. 2016).

However, elevated levels of glucocorticoid is associated with abdominal obesity, impaired glucose tolerance and blood lipid levels, which are hallmarks of DM2 (Bruehl et al. 2007). In obese males, GC sensitivity and GC feedback is impaired (Dobson et al. 2001). It has also been shown by Bruehl et al. that there is a positive association between dexamethasone-suppressed cortisol and HbA1c, which further supports the hypothesis of an interlink between a dysregulated endocrine system and hyperglycemia (Bruehl et al. 2007).

TB patients without DM2 already have higher levels of cortisol and an increased response to ACTH (Natalia Santucci et al. 2010). Cortisol levels are significantly higher in patients with moderate and advanced TB, when compared to healthy individuals and household contacts (HHCs) (Natalia Santucci et al. 2010; Natalia Santucci et al. 2011a). During anti-TB treatment, cortisol levels are elevated at diagnosis, and normalizes by month two after treatment (Díaz et al. 2015). When investigating the balance between adrenal steroids during TB treatment, there is a significant decrease in the cortisol/Dehydroepiandrosterone (DHEA) ratio, which also reach

normal levels from month two (Bongiovanni et al. 2012). DHEA, is considered to have glucocorticoid opposing effects, by inducing Th1 response and positively correlates with IFN-γ. Patients with TB have decreased plasma levels of DHEA, which decrease significantly with disease severity (Bozza et al. 2009; Bongiovanni et al. 2012).

We hypothesize that the elevated levels of cortisol in DM2 contribute to the increased susceptibility to infectious diseases particularly Mtb infection, this is with regards to the effect cortisol has on T lymphocytes. T lymphocytes is comprised of two main subsets: CD4 and CD8. CD4 cells are known as helper T cells, which can be either be Th1 or Th2. Th1 cells produce Th1-type cytokines such as IFN-γ which are responsible for proinflammatory immune responses responsible for killing intracellular pathogens (Romagnani 1999). Whereas Th2 cells produce Th2-type cytokines including IL-4, 5, 10 and 13 which have anti-inflammatory properties. When Th2 responses are in excess it will counteract Th1 responses (Romagnani 1999).

This hypothesis is supported by the anti-inflammatory effect cortisol has on the immune system by suppressing Th1 immune responses by transcriptional inhibition of pro-inflammatory cytokines (Mahuad et al. 2004; Bozza et al. 2009). Traditionally, it was thought that, GCs induce immunosuppressive and anti-inflammatory actions by promoting the shift from Th1 to Th2 activity and suppress pro-inflammatory cytokine production, immune cell trafficking and reduce the accumulation of phagocytic cells at sites of infection (Pérez, Bottasso, and Savino 2009). However, more recently it has been shown that GCs can under certain conditions have pro-inflammatory activity, thus it possess both anti- and pro-inflammatory activity. GCs can further activate the innate immune system and repress the adaptive immune response (Busillo and Cidlowski 2013).

Furthermore, under physiological conditions, cortisol can inhibit the antigen-specific immune response during TB by reducing pro-inflammatory cytokines (Mahuad et al. 2004). Such conditions thus favour mycobacterial persistence due to its inability to eliminate intracellular pathogens (Mahuad et al. 2004). Apart from inducing an effect on T cells, GCs exhibit anti-inflammatory response by increasing alternatively activated macrophages which secrete anti-inflammatory cytokines such as IL-10 (M. S. Cooper and Stewart 2009). The addition of cortisol to lipopolysaccharide (LPS)-stimulated MNs suppressed the intracellular production of TNF-α (Cheng et al. 2016).

Therefore, under certain conditions DM2 patients have elevated cortisol levels which can induce an anti-inflammatory response during an infection. This is done by upregulating Th2 cells,

alternatively macrophages and anti-inflammatory cytokines (e.g. IL-10) and suppressing Th1 which in turn decrease IFN-γ response. This would be detrimental during an Mtb infection since DM2 patients would not be able to properly control the disease.

4. Obesity and inflammation

Obesity is also associated with impairment in responses to pathogens (Grant and Dixit 2015). Obesity is a major hallmark in DM2, and contributes to low grade chronic inflammatory response seen in DM2. It is hypothesized that the increased chronic inflammatory response induced by obesity, upregulates the HPA-axis (Ceperuelo-Mallafré et al. 2016). In obese individuals, adipose tissue expands increasing its ability to act as immunological organ and controls inflammation and metabolism which contributes to the development of insulin resistance, DM2 and metabolic syndrome. Adipose tissue is being referred to as an immunological organ since numerous immune cells including T cells, B cells, neutrophils and macrophages have been identified in adipose tissue (Grant and Dixit 2015). In addition to its role as an immunological organ, it has been shown to act as an endocrine organ as well. Adipose tissue can secrete both pro- and anti-inflammatory cytokines as well as adipokines (Grant and Dixit 2015). The release of adipokines by adipose tissue is considered as the link between chronic inflammation and obesity (Reinehr et al. 2016). During obesity the number of macrophages increases in adipose tissue and accounts for 40% of total cells in adipose tissue (Lorenzo, Hanley, and Haffner 2014; Kang et al. 2016). These cells further upregulate the production of inflammatory factors. This is done by causing a phenotypic switch in macrophage polarization and upregulating pro-inflammatory cytokines such as TNF-α, IL-1β, monocyte chemoattractant protein (MCP)-1 (Kang et al. 2016). This would suggest that macrophages play an important role in the increased production of pro-inflammatory cytokines and chemokines that contribute to enhanced inflammation in adipose tissue in obesity (Kang et al. 2016). Kang et al shows that the increase in these cytokines in visceral adipose tissue precedes the infiltration of immune cells during the progression of adipose tissue inflammation in obese individuals (Kang et al. 2016). However, this effect was not seen in modestly obese humans (Kang et al. 2016). Obesity has been shown to cause central defects in T cell development that are associated with reduced production of naïve T cells by the thymus, which leads to reduced T cell receptor diversity (Grant and Dixit 2015). Increased plasma levels of IL-6 is associated in both DM2 and obesity, and have been shown to be an independent risk factor for the development of DM2 (Harder-Lauridsen et al. 2014).

TB progression however, is increased in situations of malnutrition, which lead to extreme weight loss known as cachexia (Natalia Santucci et al. 2011a). There is an inverse relationship between BMI and TB risk, suggesting that a higher BMI would be protective against TB (Anuradha et al. 2016). This would be attributed to the upregulation of Th1 and Th17 cytokines, and the lack of regulatory cytokines (Anuradha et al. 2016). Th17 cells produce pro-inflammatory cytokines such IL-17, IL-21 and IL-22, and primarily plays a role in the recruitment of leukocytes to the site of infection (Sakamoto 2012). Therefore, a paradox exists between wasting observed in TB, and obesity observed in DM2. Since, both factors are risk factors of TB progression and susceptibility.

5. Leptin

Leptin, a 16 kDa protein, is mainly synthesized by adipocytes but also from gastric and colonic epithelial cells. Leptin in conjunction with its receptor LepR are found throughout the central nervous system (CNS) and periphery where it controls appetite by reducing food intake (Mackey-Lawrence and Petri 2012). Leptin influence different tissues and systems including body lipid metabolism, hematopoiesis, pancreatic β-cell function, ovarian cell function and thermogenesis (Mackey-Lawrence and Petri 2012).

Subcutaneous adipose tissue secretes higher amount of leptin as compared to visceral adipose tissue (Ayeser et al. 2016). Leptin serum concentration is higher in females than in males with a similar body mass, thus making it sexually dimorphic (Kim et al. 2010; Natalia Santucci et al. 2011a). In obese individuals, leptin serum levels increase and is positively correlated with body mass index (BMI), HbA1c and renal function (Kang et al. 2016). Furthermore, there is an association between leptin and respiratory infections. Hyperleptinemia is associated with increased risk of death in patients with severe pneumonia (Ubags et al. 2016).

In patients with TB the level of leptin varies; when compared to HHCs the level is lower and as the disease becomes more severe the level decreases even more (Natalia Santucci et al. 2010; Natalia Santucci et al. 2011a; Yurt et al. 2013), whereas another study reported no significant difference in leptin levels in TB patients (Kim et al. 2010). In healthy individuals there is a positive correlation between BMI and leptin (Natalia Santucci et al. 2010; Natalia Santucci et al. 2011a; Yurt et al. 2013). The differences between the studies can be a consequence of the time at which blood was drawn: the lower levels were observed when blood was drawn before any TB treatment, whereas the study by Kim *et al.* blood was drawn three days after treatment. Leptin levels increase during treatment (Chang et al. 2013). Santuuci *et al.* suggest that since HHCs, who undergo a

latent sub-clinical tuberculosis infection and share the same socio-economic circumstances, show no weight loss and have normal leptin levels, that the effect is more likely due to an energy imbalance (Natalia Santucci et al. 2011b). In addition to leptin, IL-6 is associated with appetite loss. IL-6 levels were increased in TB patients. Furthermore, IL-6 known to reduce retriperitoneal fat and leptin levels. There is a negative correlation between circulating IL-6 and leptin (Natalia Santucci et al. 2011b; Kim et al. 2010).

DM2 and obesity is associated with hyperleptinemia and subsequent leptin resistance (Reis et al. 2015). Studies propose that leptin acts as neuro-inflammatory signal, which in turn attracts different immune cells and stimulate the production of IL-1β (Gorlé et al. 2016). There are leptin receptors present on microglia that regulates the synthesis of IL-1β. In both plasma and serum, leptin positively correlate with IL-1β production (Gorska-Ciebiada et al. 2016). This would suggest that hyperleptinemia and leptin resistance may lead to a higher inflammatory response in the brain (Misiak, Leszek, and Kiejna 2012).

Leptin has a direct effect on immune cells, including dendritic cells, macrophages, neutrophils, natural killer (NK), B and T cells (Reis et al. 2015). Leptin has been shown to enhance immune cell function by increasing MN and macrophages activation and improving phagocytosis during acute exposure to leptin (Ubags et al. 2016). However, as mentioned above, obesity is associated with hyperleptinemia and increased leptin consequently lead to the over production of IL-1β. Even though the pro-inflammatory cytokine IL-1β is essential in the initial control of Mtb, the continuous upregulation of IL-1β is detrimental in the control of Mtb. Excessive IL-1β induce hormonal changes by stimulating the hypothalamus resulting in increased cortisol production which influences immunity by suppressing Th1 activity. This would in turn be unfavorable for control of Mtb. However, this hypothesis is controversial since in a rat model, it has been shown that leptin neither influence diabetic hyperglycemia nor does it elevate corticosterone. Leptin instead normalized the elevated plasma corticosterone levels (Morton et al. 2015). Leptin acts as a neutrophil chemoattractant and antiapoptotic. In order for the body to maintain a pulmonary host defense intact leptin signaling is crucial (Ubags et al. 2016), however this might be lost as result of leptin resistance during obesity.

Individuals with insulin resistance (IR), produce normal levels of insulin but have a less than normal biological response, and have lower plasma leptin levels, when compared to insulin sensitive individuals. IR is associated with increased adipose secretion of IL-6 (Almuraikhy et al. 2016). In obese adolescents with DM2, they have higher levels of IL-1β, highly sensitivity C-reactive protein (hsCRP) and TNF-α compared to obese adolescents without DM2. Leptin

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negatively correlates with TNF-α (Reinehr et al. 2016). High dose of leptin activates human leukocytes in a TLR-independent fashion (Terán-Cabanillas and Hernández 2016). Recently diagnosed obese, DM2 patients present with increased TLR activation and serum TLR ligands. This response directly correlates with high SOCS3 expression in people with obesity. This has immunological implications since SOCS3 regulates type I interferon, leptin and pro-inflammatory cytokines. In peripheral tissue, SOCS3 cause insulin and leptin resistance as well as glucose intolerance (Terán-Cabanillas and Hernández 2016). The exogenous addition of high levels of leptin induce a pro-inflammatory response in PBMCs from healthy volunteers and in U937 MNs that are stimulated with LPS. Leptin upregulates TLR-2 expression in human MN without affecting TLR-4 expression (Terán-Cabanillas and Hernández 2016).

Kumar et al. proposes that the development of TB pathogenesis in DM2 individuals is associated with metabolic dysfunction due to an imbalance in the production of pro- and anti-inflammatory adipokines (Kumar, Banurekha, et al. 2014). They showed that leptin was enhanced during DM2 in both pulmonary TB and latent infection. The increase in leptin positively correlated with HbA1c. Leptin can modulate Th1 response while inhibiting the secretion of Th2 cytokines due to its structural similarity to IL-6 and IL-12 (Pavan Kumar et al. 2016). Leptin has also been reported to upregulate Th1 cytokines. Santucci *et al.* however, showed that leptin does not enhance PBMC proliferation, nor does it increase IFN-γ production thus suggesting that it has no effect on cell-mediated immune responses during a mycobacterial infection (N. Santucci et al. 2014).

Leptin is needed for an enhanced immune function during an Mtb infection by increasing MNs and macrophage activation thus improving phagocytosis or by upregulating Th1 response by increasing IFN-γ production for increased Mtb killing. However, in DM2 patients with a high BMI, the continuous upregulation of leptin leads to the increase production of IL-1β which in turn stimulated the hypothalamus and ultimately result in the elevated secretion of cortisol thus inducing the anti-inflammatory functions which is detrimental in the control of Mtb.

6. Insulin

Insulin is a 51-residue anabolic protein that is secreted by the pancreatic β-cells in the Islets of Langerhans. It is composed of two peptide chains: Chain A is composed of 21 amino acids; the B chain has 30 amino acids. Three disulfide bonds connect the chains, the mature hormone is the post-translational product of a single-chain precursor, designated proinsulin (Perlmuter et al.

2008). Key complementary functions of insulin include the stimulation of glucose uptake from the systemic circulation and suppression of hepatic gluconeogenesis, together regulating glucose homeostasis (Menting et al. 2014).

Glucose intolerance in DM2 individuals is a result of either insulin resistance, the production of normal insulin concentrations, which result in less than normal biological responses or decrease in insulin sensitivity. Insulin sensitivity refers to tissue responsiveness to insulin or how successful the receptor operates to permit glucose clearance. Therefore, poor insulin sensitivity results in the inability of glucose to be taken up by muscle tissue. Both these conditions result in hyperglycemia (Perlmuter et al. 2008). The differences between insulin resistance and insulin sensitivity may be a result of a dominance of large adipocytes in insulin resistance groups. In human fat cells, there is an elevated expression of IL-6 in insulin resistant individuals (Rotter, Nagaev, and Smith 2003). In a diabetic mouse model enhanced IL-6 production resulted in the mortality of Mtb infected mice due to an increased bacterial burden (Cheekatla et al. 2016).

In individuals with impaired glucose tolerance/ impaired fasting glucose, it have been shown that CRP, IL-6 and TNF-α are positively correlated with insulin resistance and plasma insulin concentrations (Guo et al. 2015). Insulin deficiency may cause impaired entry of Fc receptor-bound material (Almuraikhy et al. 2016). Hyperinsulinemia has been reported to increase 11β-hydroxysteroid dehydrogenase type 1 (11 β-HSD1), an enzyme which converts inactive cortisone to active cortisol, activity in human adipose tissue. Therefore, glucocorticoid induced hyperinsulinemia could account in whole or in part for increase in hepatic conversion of cortisone to cortisol via 11β-HSD1 enzyme pathway (Dube et al. 2015), and potentially supressing Th1 activity. In a study to mimic intensive insulin therapy, lymphocytes were exposed to supraphysiologic levels of insulin and induced Th2 polarization (Dube et al. 2015). Pro-inflammatory cytokines such as TNF, IL-1β and IL-6 are capable to impair insulin action and glucose uptake in peripheral tissue (Grant and Dixit 2015). Thus suggesting that insulin promotes an anti-inflammatory immune by inducing Th2 responses, this would unfavourable in the control of Mtb. However, this needs to be investigated in the context of Mtb infection.

We propose that patients with DM2 have a compromised immune system making them more susceptible to infectious disease and developing pulmonary dysfunction. Even though pro-inflammatory cytokines such as TNF-α and IL-1β are needed for Mtb control, their continuous up-regulation as observed in obese individuals could be unfavorable to control Mtb by inducing hormonal changes which result in altered immune-endocrine interactions. Increasing evidence shows that immune-endocrine responses are closely regulated and work in parallel to shape the

type of immune reaction needed upon infection. Therefore, not only immune but also endocrine factors are likely to contribute to the control or establishment of Mtb infection. We, therefore, planned a study to investigate how regulatory factors such as hyperglycemia and hormonal changes would likely alter immune function in DM2 individuals, which in turn would contribute to the increased susceptibility to TB.

Hypothesis

The central hypothesis of this study is that immune-endocrine alterations in latently infected individuals with DM2 are associated with a reduced Mtb-killing efficacy of immune cells.

Aim 1 (Study 1)

To determine the Mtb phagocytosis and or killing efficacy of PBMCs and MNs from close contacts (CCs) of TB patients with or without DM2 and its association with physiological changes characteristic of DM2.

1.1.   Objectives:

i.   To determine whether the phagocytosis of Mtb and or Mtb-killing efficacy of PBMCs and MNs is altered in patients with DM2

ii.   To establish whether possible changes in Mtb phagocytosis and or killing efficacy are associated with glycemic control

iii.   To establish whether a relationship exists between changes in Mtb phagocytosis and or killing and serum cytokine and hormone signatures in CC with or without DM2

Aim 2 (Study 2)

To identify the immune modulatory properties of endogenous hormones, *in vitro*, on PBMCs and MNs of latently infected individuals after Mtb infection.

2.2.   Objectives:

i.   Determine whether endogenous hormones alter the mycobacterial induced cytokine response of PBMCs and MNs by Luminex

ii. Determine whether endogenous hormones have an impact on mycobacterium phagocytosis and/killing in MNs and PBMCs by growth inhibition assay

iii. Determine whether endogenous hormones influence the phenotype of antigen stimulated PBMCs by flow cytometry

Chapter 2

Materials and Methods

2.1.   Study 1

2.1.1. Ethics statement

The Human Research Ethics Committee (HREC) of the Faculty of Health Sciences, Stellenbosch University, approved the study with ethics reference number (N13/05/064A). The study was conducted according to the Helsinki Declaration and International Conference of Harmonization guidelines. All study participants gave written informed consent before being enrolled into the study

2.1.2. Study participants and characterization

A total of 64 participants were enrolled from Ravensmead and Uitsig in Cape Town as part of a larger NIH funded (R01) study entitled, Altered Immune-endocrine Axis in Type 2 Diabetes and Tuberculosis Risk, which primarily enrolls close contacts (CCs) of TB patients with and without DM2. CCs are defined as individuals who have shared the same home, work or recreational space of newly diagnosed TB patients for at least a month prior to enrollment. All participants were between the ages of 30 and 65 years old, had a BMI of >20 and no current infections apart from latent TB infection (LTBI) which was confirmed with a positive QuantiFeron-Gold test. These CCs were further divided into the following groups:

- Close contacts with noDM (HC): individuals with no known DM2, HbA1c ≤ 5.6% and or normal fasting blood glucose (FBG) <6 mM.
- Pre-DM2 (preDM2): individuals with HbA1c 5.7 - 6.4% and/ FBG 6-7 mM.
- DM2 (DM2): newly identified of known DM2 patients with HbA1c of 6.5 - 7.9 % and/or FBG > 7 mM.
- Poorly controlled DM2 (pDM2): newly identified or known DM2 patients with HbA1c of ≥ 8% and/or high FBG > 7 mM.

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Study participants were excluded if they had Hb levels of <10g/L or active TB as determined by abnormal chest-x-ray, a positive GeneXpert or a positive Mycobacteria growth indicator tube (MGIT) culture. Other exclusion criteria were Human Immunodeficiency Virus (HIV) positivity, Type 1 diabetes and use illicit drugs more than once a week or on any immunosuppressive drugs. Pregnancy or other medical conditions including cancer, severe systemic conditions, chronic bronchitis, emphysema and asthma requiring steroid therapy. Participation in a drug or vaccine trial.

2.1.3. Sample collection and processing

Blood was collected, from fasting participants, in 9 ml sodium heparin tubes (Lasec, Cape Town, SA), 3 ml lithium heparinized tubes (Lasec), 3ml serum-separating tubes (SST tubes; BD Biosciences, Franklin Lakes, NJ, USA) and 3ml Ethylenediaminetetraacetic acid (EDTA) tubes (BD Biosciences) between eight and nine AM. Clinical information on age, sex, weight and height were recorded to determine BMI as indicated in Table 1. Sputum samples were collected for a GeneXpert test (Cepheid, Sunnyvale, CA, USA) and MGIT (BD Biosciences) culture. EDTA and SST tubes were sent to the National Health Laboratory Services (NHLS) for full blood and differential cell counts, cortisol, insulin and HbA1c measurements.

2.1.4. Whole blood interferon-gamma release assay

Interferon-Gamma Release Assays (IGRAs) are tests performed on whole blood that aid in the diagnosis of Mtb infection (active disease and LTBI). One ml of whole blood, collected in lithium heparinized tubes, was added to QuantiFERON (QFN)-TB Gold nil, antigen (ESAT-6, CFP-10 and TB7.7) and mitogen tubes (Cellestis Limited, Carnegie, Victoria, Australia) respectively and was processed as per manufacturer instructions. Plasma was collected after 18-20 hours and stored at -80 °C until the QFN enzyme-linked immunosorbent assay (ELISA; Cellestis Limited) was performed. Briefly, a conjugated monoclonal antibody to IFN-γ and QFN supernatant were added to the ELISA wells and mixed thoroughly which was then incubated at room temperature (RT) for 2 hours. The wells were washed 6X with wash buffer, afterwards the enzyme substrate solution was added and the plates were incubated for 30 min at RT in the dark. After incubation, the enzyme stopping solution was added to stop the reaction. The absorbance was recorded at

450 nm on the Microplate reader (Bio-Rad Laboratories, Hecules, CA, USA). All standard curves had an r^2 of ≥ 0.98. The IFN-γ concentration (IU/ml) was quantified by comparison with the appropriate recombinant standard. The QFN ELSIA outcomes are determined by subtracting the IFN-γ concentration of the nil from the TB antigen and three outcomes occur: negative (Mtb infection not likely), positive (Mtb infection likely) and indeterminate (results are indeterminate for TB-Antigen responsiveness). This was dedtermined by QunatiFERONE Gold-In-Tube Package version 2.5 (Cellestis Limited). The absolute IFN-γ concentration obtained from the antigen-stimulated tube was used during downstream data analysis. Spearman correlations were performed between absolute IFN-γ concentration and DM2 associated variables.

2.1.5. Preparation of H37Rv Mtb

The laboratory Mtb strain, H37Rv, was used in all experiments. For in vitro cells infection, frozen H37Rv Mtb was prepared. One ml of H37Rv Mtb was added to 5 ml of Middlebrook 7H9 agar-Tween (7H9T) (BD Biosciences) with Oleic Albumin Dextrose Catalase Growth Supplement (OADC) (BD BioSciences) in a flask. The culture was incubated at 37°C until the OD reached between 0.5 and 0.8. Two ml of Mtb stock was transferred to 50 ml of 7H9T-OADC and incubated at 37°C until the OD was 0.6. The Mtb culture was then distributed into 50 ml tubes containing 3-5 sterile beads and spun down at 2 500 x g for 10 min. The supernatant was removed and the pellet washed with 7H9T and resuspended with less than 50% of the original volume using 7H9T. To remove any clumps, the culture was pulse-votex for 5 seconds. Afterwards the culture was sonicated for 30 seconds. The culture was then centrifuged for 300 x g for 5 min without breaks to separate clumped Mtb (pellet) from the non-clumped Mtb (supernatant). The supernatant was pooled and vortexed. Glycerol (Merk, Kenilworth, NJ, USA) was added to a final concentration of 10%. A frozen aliquot of the stock was thawed and the colony forming units (CFUs) enumerated to determine the concentration of the stock. Culture viability was further determined by performing a MGIT (BD BioSciences) (7 ml MGIT tube + 1.6 ml growth supplement-antimicrobial agent (PANTA) mixture + 100 µl Mtb) of the culture stock on the day of freezing and every week during the first month of freezing.

2.1.6. Growth inhibition assay

i.   Plasma collection and PBMC isolation

Within 2 hours after blood collection tubes arrived in the laboratory, the sodium-heparinized blood was pooled in a 50 mL tube (Lasec) and was spun down at 800 x g for 12 minutes at 20°C with the acceleration at 9 and the breaks off. The upper portion containing the plasma was transferred to a 15 ml tube (Lasec) and placed on ice for at least 10 min after which it was spun at 2 500 x g for 15 min at 4 °C to allow clots to settle at the bottom of the tube. After plasma was removed, the remaining plasma (about 1 cm above white blood cell interphase) and the white blood cell interphase together with roughly 1 cm of red blood cells, below the white blood cell interphase, it was collected in separate 50 ml tube and mixed well to avoid white blood cell clumps. PBS (Lonza, Basel, Switzerland) containing 1 mM EDTA (Sigma-Aldrich, St Louis, Missouri, USA) and 1% Human serum albumin (SEH) were added to the cells to replace the removed plasma. SEH was added to the 35 ml mark and mixed gently by inversion. The cells were layered over 15 ml Ficoll-Paque PLUS (GE Health, Piscataway, NJ) (density 1.077) and centrifuged at 600 × g for 30 min at RT (19- 23 °C) with the breaks and acceleration off. PBMCs recovered from the interphase after the gradient centrifugation were washed with 50 ml cold RPMI-HEPES (Sigma-Aldrich) twice by first centrifuging it at 600 x g for 4 minutes and then at 150 x g for 8 min with the centrifuge set at (accel = 9; brake = 0), 4°C. The media was removed and the pellet was replenished with 5 ml of RPMI (Biowest, Nuaillé, France) containing 2 mM Glutamine (Glut) (Sigma-Aldrich) and 100U/ Penicillin (Pen) (Sigma-Aldrich) (RPMI+Glut+Pen). The cell count was determined by diluting cells 1:10 in 10% Tryphan Blue (Sigma-Aldrich) (in PBS) and loaded on Countess Cell Counting Chamber Slides which were designed for Countess Automated Cell counter (ThermoFisher, Waltham, Massachusetts, USA). PBMCs were cultured in RPMI+Glu+Pen with 20% autologous plasma in triplicate in Falcon 96-well round bottom plates (The Scientific Group, Johannesburg, SA) at a concentration of 2 x 10^5 cells/well in a final volume of 200µl or a screw cap tube (Whitehead Scientific (Pty) Ltd, Cape Town, SA) at a concentration of 6 x 10^5 cells/well in a total volume of 600µl. The culture was incubated overnight at 37 °C in 5% CO_2.

ii.   Monocyte (MN) isolation

The remaining PBMCs were spun down at 300 x g for 10 min, and MNs separated by negative selection using MACS microbeads (Miltenyi Biotec, Bergisch GladBach, Germany) coated with anti-lymphocyte antibodies including T cells, B cells, dendritic cells, NK cells and basophils as per manufacturer's instructions. Briefly, PBMCs were resuspended in MACS Buffer (PBS containing 0.05% BSA and 2 mM EDTA) (Miltenyi Biotec), FcR and biotin-Ab cocktail (Miltenyi Biotech) and incubated for 5 minutes at 4°C. Afterwards MACS buffer and anti-biotin microbeads were added, mixed well and incubated for 7 min at 4°C. The cocktail was passed through large MACS column (Miltenyi Biotech) placed in a magnetic separator. The column was washed 3X with Rinsing Buffer (PBS with 2 mM EDTA;Miltenyi Biotech). The cell suspension that passed through the column was collected in a 15 ml tube and spun down 300 x g for 7 min. The supernatant was decanted and the pellet was resuspended in 1 ml RPMI+Glu+Pen, the cell count was determined as described above. The MNs were cultured in RPMI+Glu+Pen with 20% autologous plasma in triplicate in 96- flat bottom poly-D-lysine coated plates (The Scientific Group) at a concentration of 1.3 x 10^5 cells/well in a final volume of /200µl and incubated for 16 h at 37 °C in 5% CO_2.

iii.   H37RV Mtb infection

After the overnight incubation PBMCs and MNs were washed once with RPMI-HEPES (Sigma-Aldrich) and infected with H37Rv Mtb at a final concentration of 3.25 x 10^6 CFU/mL multiplicity of infection (MOI) (MOI 1:1) in RPMI+Glu+20% autologous plasma for 2 hours at 37 °C in 5% CO_2. After infection, unbound Mtb was washed off with RPMI-HEPES twice and the cells were harvested a) immediately, b) 1 day post infection and c) 3 days (for MNs) or 6 days (for PBMCs) post infection for CFU determination. Culturing of cells for 1, 3 or 6 days was done using (RPMI + Glu containing 20% autologous plasma. Seventy-two hours post infection, 50% of supernatant of PBMCs was removed and replaced with fresh RPMI-Glu containing 20% autologous plasma. This was done to ensure sufficient nutrients are available to cells to survive until 6 days post infection without removing all the cytokines produced by the cells in culture needed for cellular growth and differentiation. Cells were harvested by lysing adherent cells with 100µl of 0.05% sodium dodecyl sulfate (SDS;Sigma-Aldrich) per well for 5 min at RT and transferring the lysate to 2ml centrifuge tubes (The Scientific Group) containing an equal volume of 7H9 (BD Biosciences) agar. Serially diluted lysates were plated in duplicate on Middlebrook 7H11 (BD Biosciences) agar plates supplemented with 10%

OADC (BD Biosciences) and 0.5% glycerol (Merk) and incubated at 37°C. Colony forming units (CFU's) were determined after 3-4 weeks. CFU counts between 20-200 were deemed accurate to eliminate either under counting the true number of CFUs as a result of overcrowding (higher than 200 CFUs) or over estimating a count (lower than 20) CFU's counts outside this range were not taken into account, and CFU/mL was calculated.

2.2. Study 2

2.2.1. Study participants and sample collection

Blood from fasted, healthy community donors were collected in 9 ml sodium heparinized tubes (Lasec) and 3 ml lithium heparin (Lasec) were collected before 9:30 AM in the morning, and processed within 2 hours after arriving in the laboratory. All the participants were tested for latent Mtb infection as described in section 2.1.4.

2.2.2. Optimization

PBMCs were isolated from the blood of a healthy participant as indicated in 2.1.6. with the exception that cells were cultured in either AIM-V (BD Biosciences) media or RPMI (Biowest) with 2 mM Glu (Sigma-Aldrich) and 10% heat inactivated human serum (hiHS: Sigma-Aldrich). Heat inactivation was done to inactivate complement activity in the serum. After cells were seeded overnight, the cells were washed once with RPMI-HEPES (Sigma-Aldrich) and infected with *H37Rv* Mtb with a MOI of 1:1 for 2 hours. After the cells were washed, they were harvested immediately, at day 1 and day 3 post infection. After the wash step the media was replenished for cells incubated until the later time points. Harvesting include adding 100 µl of cold 2mM EDTA (Sigma-Aldrich) to each well. The cells were incubated for 10 min at 4 °C. Cells were transferred to round bottom tubes (The Scientific Group), and spun down at 250 x g for 5 min. The cells are washed with 1 ml of PBS (Lonza) and spun down at 250 x g for 5 min. Cells were then resuspended in PBS (Lonza) and stained with a 1:100 live/dead aqua (Biolegend) fluorescent dye and incubated for 30 min, RT in the dark. Cells were then washed with PBS (Lonza), and then fixed with 1.0% formaldehyde (Merk) for 15 min, RT. Cells were washed with FACS buffer, and then resuspended in 150ul FACS buffer. The acquisition of the cells was performed using the FACS canto II instrument (BD Biosciences) equipped with FACS Diva software (BD Biosciences), and analyzed using FlowJo software (Treestar, San Carlos, CA).

2.2.3. Growth inhibition

i.   PBMC and MN culture and hormone treatment

PBMCs and MNs were isolated as indicated in 2.1.6. After cell counts were determined, PBMCs and MNs were cultured in triplicate in RPMI+2 mM Glut+ 10% heat inactivated (hiHS) (Sigma-Aldrich), to reduce serum complement activity, in Falcon 96-well round bottom plates (The Scientific Group) (2 x 105 cells/200μl well) and Falcon 96- flat bottom plates (The Scientific Group) (1.3 x 105 cells/200μl well) respectively. Cell cultures were seeded overnight at 37 °C in 5% CO_2 under the following conditions: either with two different concentrations; a low (10^{-9} M) or high (10^{-7} M) concentration, of cortisol (Sigma-Aldrich), leptin (Sigma-Aldrich) or insulin (Sigma-Aldrich) or without any hormones (controls).

Prior to the use in culture, cortisol was dissolved in ethanol (EtOH) (Merk), insulin in hydrochloric acid (HCl) (Merk) and leptin in PBS (Lonza). Cortisol and insulin stocks were stored at -20 °C and leptin at -80 °C.

ii.   H37RV Mtb infecton

Similar to study one, PBMCs and MNs were washed with RPMI-HEPES (Sigma-Aldrich) and infected with H37Rv Mtb at a final concentration of 3.25 x 10^6 CFU/ml (MOI 1:1) in RPMI+Glu+10% hiHS for 2 hours at 37 °C in 5% CO_2. After the infection, cells were washed twice to with RPMI-HEPES to remove unbound Mtb. Cells were harvested a) immediately, b) 1 day post infection and c) 3 days post infection for CFU determination for both cell types. Again after washing the cells the media (RPMI-Glu-10% hiHS) was replenished in wells containing cells that were incubated until the 1 day and 3 days post infection time points. With the exception that the media either contained one of the two concentrations (10^{-9} and 10^{-7} M) of either cortisol, insulin or leptin. The harvesting of cells and determination of CFUs remained the same as study 1.

2.1.1. Milliplex® Map Kit: Human Cytokine/Chemokine detection

The Merck Millipore Luminex technology (Miltenyi Biotec) was used to determine the cytokine production during the growth inhibition assay of PBMCs. Supernatants of uninfected PBMCs were included as control. Supernatants of uninfected PBMC of 3 study participants were pooled

together, due to the space and cost constraint of the luminex assay. While the supernatants of Mtb infected PBMCs of study participants were analyzed separately. The Human Cytokine/Chemokine detection assay (Miltenyi Biotec) was used to detect the following cytokines in undiluted samples: granulocyte macrophage colony-stimulating factor (GM-CSF), fibroblast growth factor (FGF)-2, vascular endothelial growth factor (VEGF), interferon (IFN)-γ, IFN-α, interleukin (IL)-1β, IL-1RA, IL-5, IL-6, IL-8, IL-9, IL-10, IL-13, IL-17A, tumor necrosis factor (TNF)-α, TNF-β, interferon gamma-induced protein (IP)-10. The assay was done according to manufacturer's instructions. Briefly, 200 µl of wash buffer was added to each well of the plate to pre-wet the plate. The wash buffer was decanted and 25µl of standard, assay buffer (0 pg/ml and sample wells) and controls were added to the appropriate wells. This was followed by adding 25 µl of antibody-immobilized beads to all the wells. The plate was sealed and wrapped in foil and placed on a plate shaker for 16 h at 4°C. After the overnight incubation, the contents were removed and the plates were washed 2X with 200 µl of wash buffer. 25 µl of detection antibody was added to each well, and the plate incubated for 1h at RT on a plate shaker. 25 µl of Streptavidin-Phycoerythrin was added to each well, and again incubated for 30 min at RT on a plate shaker. After the last incubation, the contents were removed and the plate was washed 2X with 200 µl wash buffer and 150 µl of sheath fluid was added to the wells. Two quality controls included in each kit were run in duplicate on the plate. Levels of all analytes in the quality controls were within the expected ranges. A standard curve ranging from 3.2 pg/ml to 10 000 pg/ml was used for all analytes. Analyte concentrations were determined using the Bio-Plex 200 platform (Bio-rad, Hercules, CA, USA). The Bio-Plex Manager Software version 6.1 was used to analyze the data.

2.1.2. Flow Cytometry

PBMCs were isolated, treated with or without different concentrations of hormones and infected with Mtb as described above. After 1 day and 3 days post infection, cells were resuspended in cryomedia (fetal bovine serum (FBS) (Sigma-Aldrich) with 10% dimethyl sulfoxide (DMSO) (Merk) and stored away at -80°C until all samples were collected. After thawing the PBMCs, 1 ml of warm thawing media (RPMI + 10% FBS +1% L-Glut) were added to the cryovial in a drop wise manner and cells were subsequently transferred to a 15 ml tube containing 7 ml of thawing media. The cell suspension was mixed thoroughly and then spun down for 10 min at 250 x *g*, afterwards the pellet was washed with 10 ml of thawing media and spun down for 10 min at 250 x *g.* The pellet

was resuspended in 1 ml of PBS, spun down at 250 x *g* and the supernatant decanted. Cells were resuspended in PBS and stained with 1:50 live/dead Zombie Aqua Dye (viability stain, Biolegend) and incubated for 15 min, RT in the dark. Cells were washed twice with FACS buffer (PBS+2% FCS). Thereafter, the cells were stained with 50 µl pre-titrated antibody mix containing antibodies for the following markers: CD3-PerCP (1:40; BD Biosciences), CD4-BV421 (1:40; Biolegend), CD45RA-APC (1:80; Biolegend), CD45RO-PE-Cy7 (1:80; Biolegend), CD38-APC-Cy7 (1:40; Biolegend), CD127-FITC (1:10; Biolegend) and CD197-PE (1:10; Biolegend). After a 1 hour incubation at 4 °C (in the dark) cells were washed twice with 250 µl FACS buffer and fixed with 100 µl of 1% formaldehyde for 15 min at RT. Cells were washed once with FACS buffer, and resuspended in 200 ul FACS buffer. The acquisition of the cells was performed using the FACS canto II instrument (BD Biosciences) equipped with the FACS Diva software (BD Biosciences), and analyzed using FlowJo software (Treestar, San Carlos, CA). Further details on the gating strategy is shown on Figure 16.

2.2. Statistical analysis

A student's t test was used to determine statistical differences between two groups. A value of p < 0.05 indicated significant difference. Correlations were done with spearman rank test. Demographic and biochemical parameters of CCs with and without DM2 were done using one-way ANOVA with a Bonferroni Post hoc test. Analyses data were done by making use of the GraphPad prism version 5.00 for Windows (GraphPad Software, San Diego California, USA). To determine phagocytosis and killing over time, analysis was done using a mixed-model repeated-measures analysis of variance (ANOVA) with a Fisher LSD post hoc test by using Statistica version 11 (StatSoft, Tulsa, Oklahoma, USA). For the Luminex and flow cytometry analysis for comparing the effect of hormone treatment within one time point, we made use of non-parametric Kruskal-Wallis test with a Dunns Post hoc test using GraphPad prism version 5.00.

Chapter 3

3. Results Study 1

In this chapter, we aim to investigate Mtb phagocytosis and or Mtb growth containment of PBMCs and MNs from CCs of TB patients with or without DM2 and its association with physiological changes such as hyperglycemia, chronic inflammation and dysregulated hormones, characteristic of DM2. To test this, we isolated PBMCs and MNs from CCs with and without DM2 and infected these cells with Mtb for 2 hours. After infection, unbound cells were washed and then harvested either immediately (to determine the uptake), 1 day, 3 days and 6 days post infection (to determine killing efficacy) by lysing cells. Lysates were then plated to determine CFUs. When comparing CFU numbers between groups at a time point, a higher number of CFUs were indicative of increased uptake (2 hours) or bacterial burden and/ or decreased bacterial killing (day 1, 3 and day 6) thus poor control of Mtb by the immune cells. We went further and correlated CFUs with physiological characteristics such hyperglycemia, full blood and differential cell counts, cortisol, insulin and absolute IFN-γ concentrations. We also determined the differences of these characteristics among the four patient group: healthy, preDM2, DM2 and poorly controlled DM2 participants (abbreviated as HC, preDM2, DM2 and pDM2, respectively, in the figures below). In addition, we grouped Healthy and preDM2 (HH) and DM2 + pDM2 (DM).

3.1. Study participant characteristics

From the 64 enrolled CCs of TB patients, 3 individuals had a positive GeneXpert and were excluded from analysis since this was an indication of active disease. From the remaining participants, 58 were IGRA positive, and 3 were IGRA negative. The 3 IGRA negative individuals were included in the data analysis. The characteristics of the 61 participants are illustrated in table 1.

Table 1. Baseline characteristics of study participants.

	HC	preDM2	DM2	pDM2	p-value
N	25	15	14	7	
Female (n)	15	10	12	7	
IGRA neg (n)	1	2	0	0	
Age (years)[*]	44 ± 7 [a]	50 ± 9 [ab]	52 ± 9 [b]	55 ± 8 [b]	0.0067
HbA1c (%)[*]	5.1 ± 0.4 [a]	6.0 ± 0.2 [b]	6.9 ± 0.5 [c]	11.2 ± 2.3 [d]	<0.0001
BMI (kg/m^2)[*]	27.8 ± 8.6	27.3 ± 8.5	32.1 ± 9.8	29.6 ± 8.5	0.3010

[*] Values are expressed as mean ± SD.
P values were determined one-way analysis of variance, followed by a Bonforoni post hoc test.
Letters a-d indicate statistical significance

## 3.2. Poor intracellular bacterial control of PBMCs and monocytes from patients with DM2

The innate immune system relies on phagocytic cells such as monocytes to protect the host from opportunistic pathogens (Sherwood and Toliver-Kinsky 2004). Individuals with diminished phagocyte function have difficulty to clear or control bacterial infections. This is problematic especially for individuals with DM2 who have been shown to have diminished phagocytic activity (Lecube et al. 2011; Restrepo et al. 2014; Gomez et al. 2013), but how that relates to Mtb infection in LTBI individuals needs further investigation. To determine whether phagocytosis and/ or killing of Mtb were altered during diabetes, we investigated the uptake and growth of Mtb in PBMCs and monocytes isolated from the blood of CCs of TB patients with and without diabetes. The CFUs observed at the 2 hour time point were indicative of uptake (phagocytosis), and change in CFUs at day 1, 3 (MNs) and 6 (PBMCs) gave an indication of bacterial burden and/ or killing. A higher number of CFUs would suggest increased bacterial burden and/ or decreased killing thus poor control of Mtb. For simplicity, we would refer to CFUs at day 1, 3 and 6 as bacterial burden. The CFU data were plotted either as raw or as a fold growth when compared to the 2 hour time point.

When investigating Mtb control in PBMCs, bacterial burden increased significantly from day 1 to day 6 in both HbA1c 6.5 – 15.2 % (DM) and HbA1c 4.0 - 6.4% (HH) groups (Figure 3 A). We continued and subdivided the HH group into individuals with normal HbA1c (HC) (HbA1c 4.0-5.7%) and those who are at high risk of developing DM2 i.e preDM2 (HbA1c 5.8-6.4%) to see

whether the same pattern would continue. Interestingly, in both the DM2 and preDM2 groups there were a significant increase in bacterial burden from day 1 to day 6 (Figure 3 B). To normalize for any variation that may been caused by uptake of the bacterium by PBMCs we further investigated the fold growth over time. We found a significant increase from day 1 to day 6 in the DM group when compared to HH (Figure 3 C), and the pattern only continued in the DM2 and not the preDM2 group (Figure 3 D).

Given the complexity of the interaction between different cell types in PBMCs, we further investigated whether the phagocytic activity, of a single cell type, MNs, were altered in DM2. Similar to PBMCs, there was a significant increase in the bacterial burden from day 1 to day 3 in both HH and DM (Figure 4 A). We again assessed whether a preDM status would influence the phagocytic activity differently, interestingly a significant change in bacterial burden was only observed in the two extreme groups; HC and DM2 (Figure 4 B). Unlike PBMCs, when comparing the fold growth of MNs between HH and DM, both groups experienced a significant increase in bacterial burden from day 1 to day 3, with the DM group having a significantly higher bacterial burden at day 3 (Figure 4 C). When we separate the preDM2 from the HH group, we did not observe a significant change in bacterial burden over time in this group. However, the bacterial load in the DM2 groups was significantly higher than the preDM2 group (Figure 4 D).
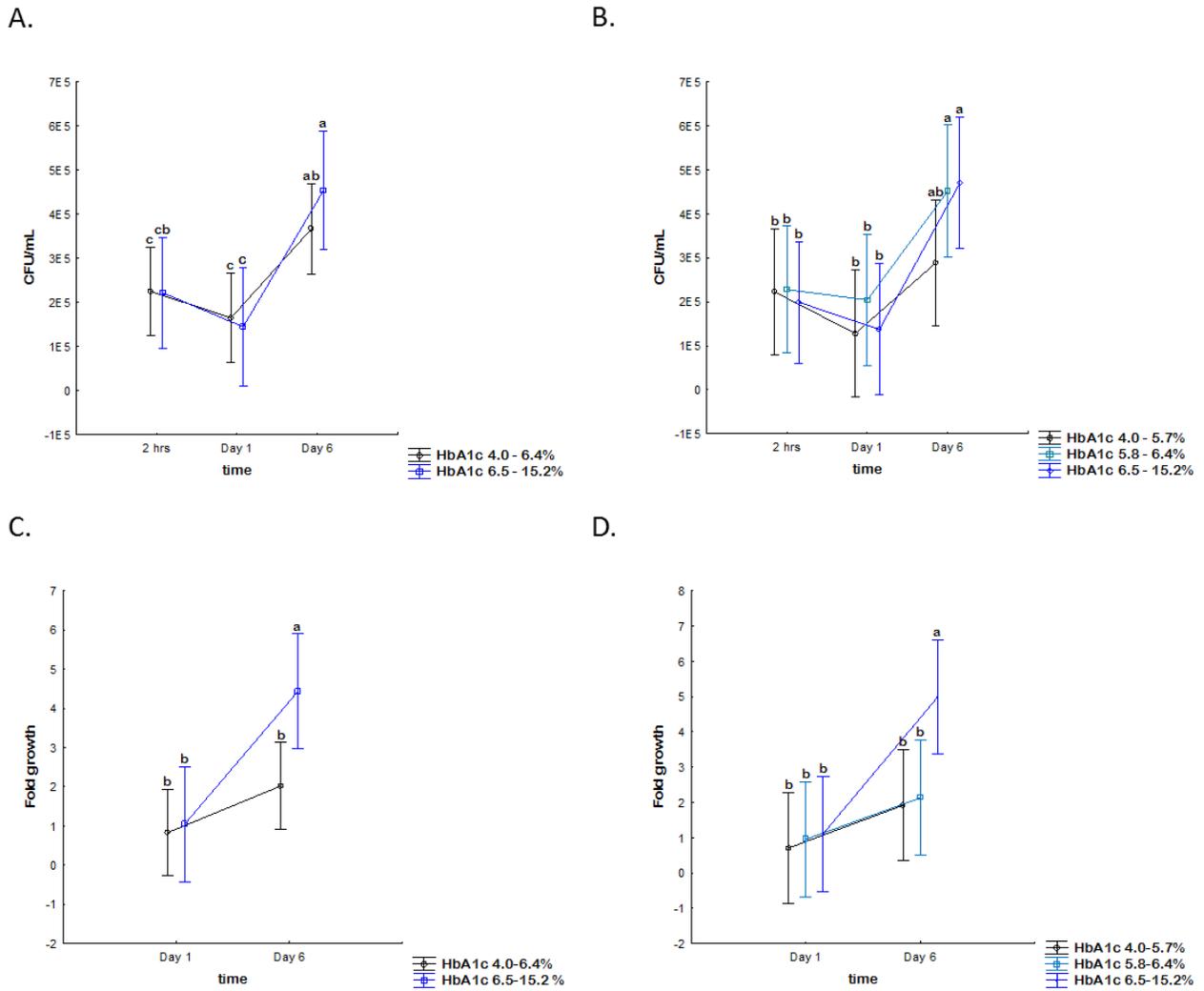
Figure 2. The effect of diabetes on PBMCs control of Mtb. PBMCs from individuals with HbA1c 4.0-6.4 % (n=27), and HbA1c 6.5-15.2% (n= 15) (A and C), and HbA1c 4.0-5.7 (n= 17), HbA1c 5.8-6.4% (n= 10) and HbA1c 6.5-15.2 % (n= 15) (B and D) were infected with Mtb for 2hrs and were harvested at three time points (2hrs , day 1 and 6) post infection. CFUs were enumerated after 3 weeks. Data was analysed with a mixed model repeated measure ANOVE with a Fisher LSD Post-hoc test. Data is represented as LS means with 95% CI. Letters a-d indicate statistical significance when comparing the groups at a particular time point as well as comparing the groups over time. Values with the same letter are not statistically different from each other. A p-value of <0.05 was considered as significantly different.

A.

B.

C.

D.
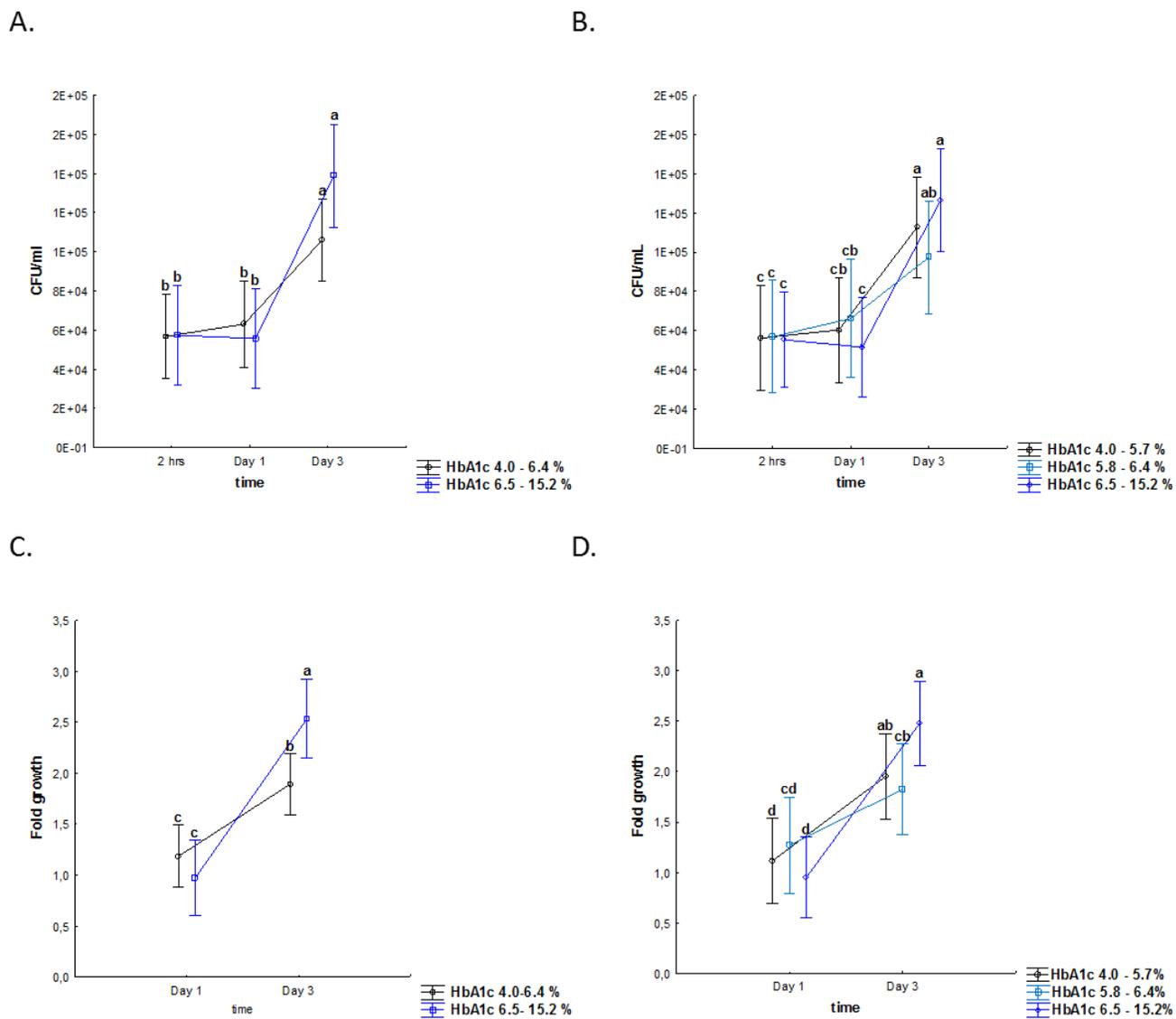
Figure 3. The effect of diabetes on MNs control of Mtb. MNs from individuals with HbA1c 4.0-6.4 % (n=19), and HbA1c 6.5-15.2% (n= 12) (A and C), and HbA1c 4.0-5.7 (n= 13), HbA1c 5.8-6.4% (n= 6) and HbA1c 6.5-15.2 % (n= 12) (B and D) were infected with Mtb for 2hrs and were harvested at three time points (2hrs , day 1 and 6) post infection. CFUs were enumerated after 3 weeks. Data was analysed with a mixed model repeated measure ANOVE with a Fisher LSD Post-hoc test. Data is represented as LS means with 95% CI. Letters a-d indicate statistical significance at each time point between different groups. Values with the same letter are not statistically different from each other.

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3.3. Poor intracellular bacterial control of PBMCs and monocytes from patients with DM2 were associated with glycemic control and white blood cell counts

When adjusting for any variation caused by uptake, we found that PBMCs and MNs of individuals with DM2 had poor intracellular bacterial control as indicated in figure 3C and 4C. We then investigated whether there was an association between the CFU data and glycemic control. To examine the association between bacterial control in PBMCs and MNs with glycemic control, we correlated the raw CFU data with HbA1c and FBG and performed spearman correlations. Interestingly, we observed a significant positive correlation between CFU of PBMCs at 2h and FBG (p=0.01, r=0.63) in the DM group (Table 2), and only a trend for a positive correlation with HbA1c (p=0.08, r=0.46) (data not shown). The association remained consistent when separated into DM2 (p=0.039, r=0.58) and preDM2 groups (p=0.023, r=0.72), the pDM2 only had one participant thus statistical analysis was not done, the latter group having a stronger positive correlation with FBG (Table 2). On day 1 and day 6, there were no significant correlation between FBG and CFUs in all groups (data not shown). This would suggest there was an association between FBG and uptake. Similarly, a significant positive correlation (p=0.013, r=0.58; p=0.024, r=0.67) was seen between the CFUs at 2h (MNs) and CFUs at day 3 and FBG in DM patients respectively (Table 2). However, on day 1 there was no association between CFUs and FBG (data not shown). There was a negative correlation between CFUs at day 3 in MNs and FBG (p= 0.028 r= -0.50) in HH (Table 2). This trend continued when individuals with preDM2 were removed from the analysis (p= 0.06, r= -0.58) HC (Table 2). FBG only has an association with uptake and killing in DM2 MNs, and only killing in HC MNs.

Poor bacterial control in PBMCs and MNs of individuals with DM2 was observed, therefore we investigated whether there was a relationship between CFUs observed and the frequencies and absolute numbers of other immune cells in the whole blood. To test this, we correlated the CFU data with different cell types. We observed that CFUs of PBMCs of HHs at 2h positively correlated with the white cell counts (WCC) (p=0.0029, r=0.56) (Figure 5 A and B), whereas in DM patients it positively correlated with neutrophil numbers (p=0.026, r=0.57) (Figure 5 C). Upon further analysis, monocyte numbers positively correlated with the CFUs in PBMCs at 2h in HH (p= 0.005, r=0.63) (Figure 5 E and F).

We went further and investigated how CFU data in MNs correlated with the different cell types. We observed that CFUs of MNs at 2h in the HH group positively correlated with eosinophil (p= 0.044, r=0.48) (Figure 5 G and H) and basophil numbers (p=0.043 r=0.48) (Figure 5 I and J) respectively and negatively correlated in DM individuals, however this was not statistically significant.

3.4.   High white blood cell counts in individuals with diabetes

Diabetes is a chronic inflammatory disease therefore, we compared the different white blood cell counts between individuals with and without DM2. We saw that individuals with DM had significantly higher white cell count (WCC) and neutrophil counts when compared to HH (Figure 6 A and C). When separating the groups into preDM2 and pDM2 we saw that individuals with normal HbA1c had less WCC compared to DM2 and pDM2 patients (Figure 6 B). The preDM2 group had less WCC compared to pDM2 patients. Furthermore, DM2 and pDM2 had significantly higher neutrophil counts compared to normal HbA1c participants (Figure 6 D). Interestingly, despite the high white cell count, individuals with DM had significantly lower monocyte number in the blood compared to individuals with without DM (Figure 6 E). When the participants are separated into the 4 groups, the significance was lost (Figure 6 F). No differences were found in lymphocyte counts and basophil percentages between HH and DM patients (Figure 6 G and H).

3.5.   High insulin levels was associated high bacterial burden in individuals with diabetes

We further investigated whether hormones such as insulin and cortisol had an association with high bacterial burden observed in PBMCs and MNs. As mentioned in introduction, these hormones have an influence on immune function thus potentially influence bacterial burden or control. We measured the levels of insulin and cortisol in the serum of both individuals with and without diabetes. We saw that individuals with DM have a significantly higher level of insulin than HH (Figure 7 A). No differences were seen when the groups were further divided into DM2 status (Figure 7 B). We saw that cortisol levels were the same in individuals with and without DM (Figure 7 C-D).

Table 2: The association between phagocytosis and killing of Mtb in PBMCs and MNs with glycemic control. Spearman correlations between the CFUs of PBMCs and MNs with FBG.

	HH		DM		HC		preDM2		DM2		pDM2	
	p value	R	p value	R	p value	R	p value	r	p value	r	p value	r
PBMC 2h CFU/ml	0.45	0.14	**0.011**	0.63	0.67	0.11	**0.023**	0.72	**0.034**	0.58	#	
MN 2h CFU/ml	0.83	-0.054	**0.013**	0.58	0.41	0.26	0.26	0.34	**0.0062**	0.57	#	
MN D3 CFU/ml	**0.028**	-0.50	**0.024**	0.67	0.06	-0.53	0.84	-0.04	**0.02**	0.74		

\# Not enough XY pairs to perform statistical analysis
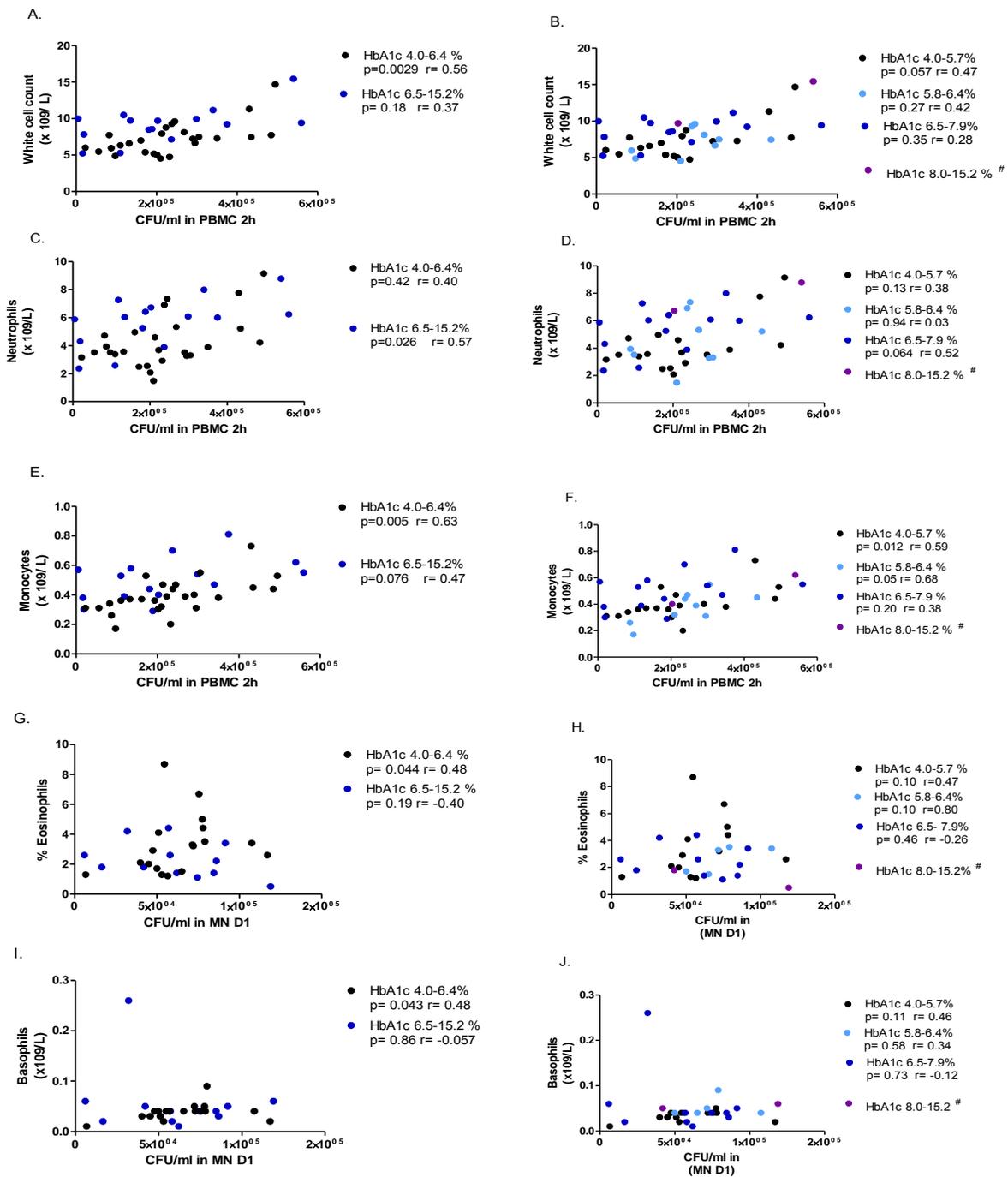
Figure 5. The association between phagocytosis and killing of Mtb in PBMCs and MN with different cell types.

Spearman correlations between the CFUs of PBMC at 2h and white cell count (A-B), neutrophils (C-D) and MN (E-F); as well as between CFUs of MNs at D1 and eosinophils (G-H) and basophils (I-J).

 # Not enough XY pairs to perform statistical analysis.

46
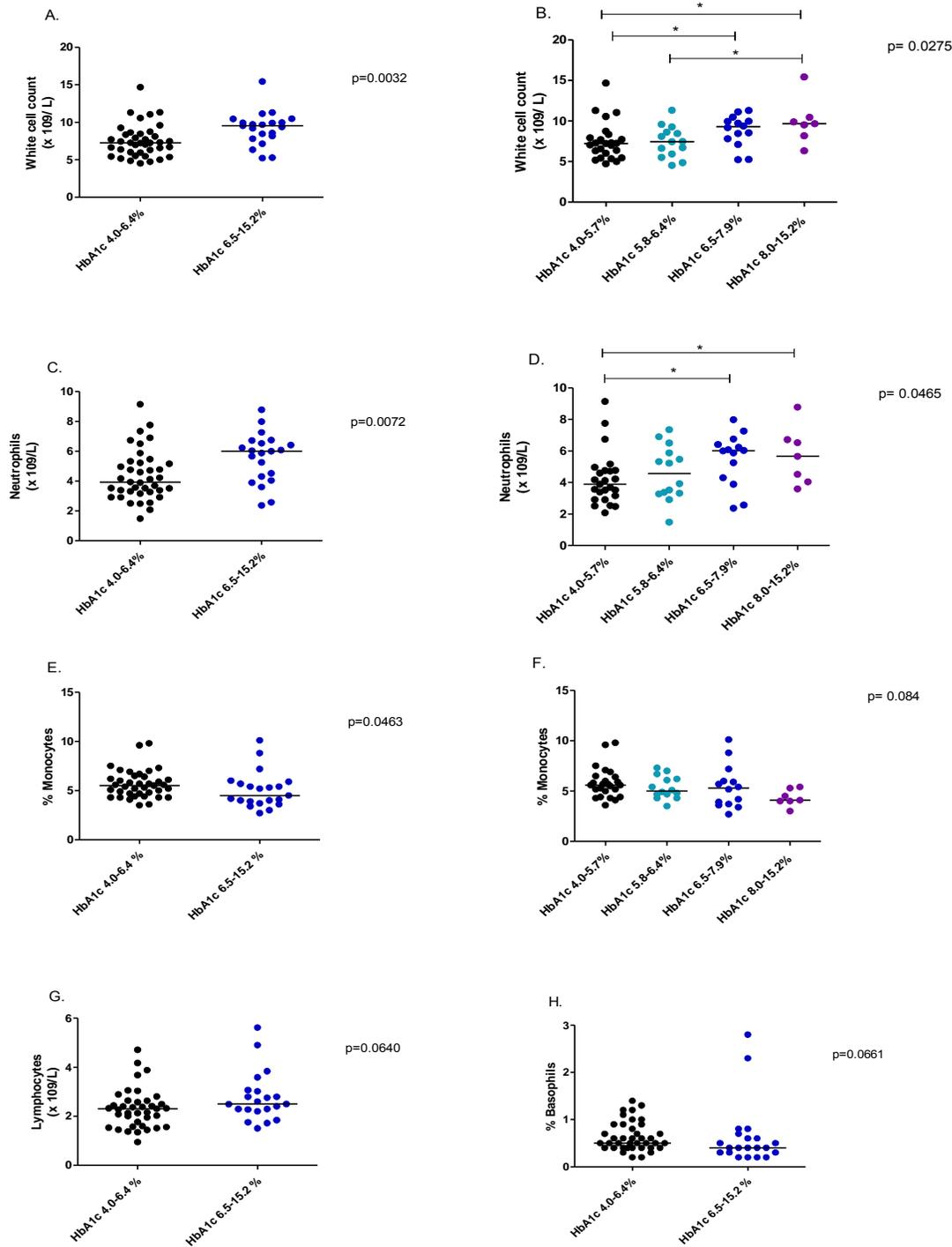
Figure 6. High white blood cell counts in individuals with diabetes. The cell counts were determined for white cell count (A-B), neutrophils (C-D), monocytes (E-F) and lymphocytes (G) and Basophils (H). In A, C, E, G and H, a Mann Whitney U T tests was performed, and in B, D and F a Kruskal-Walis tests with Dunn's post-hoc test was used: Comparing all pairs of columns. A p-value of < 0.05 was regarded as statically significant.
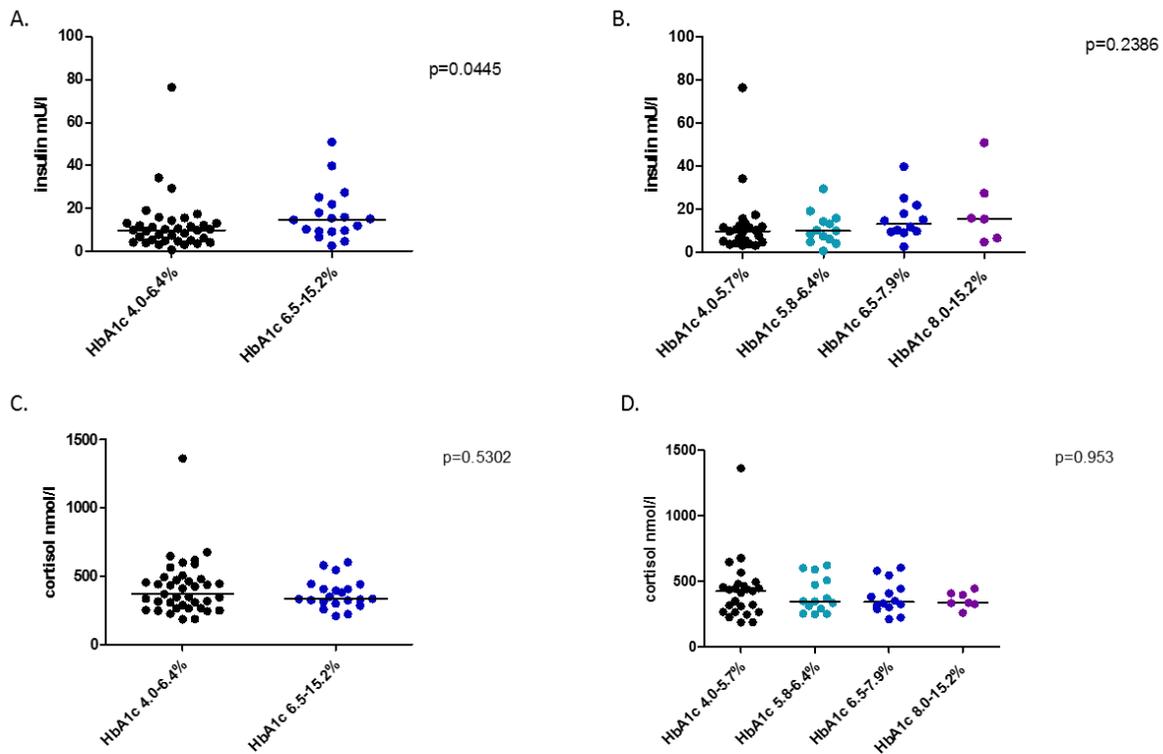
Figure 7. Higher insulin levels has an association with high bacterial burden in individuals with diabetes. The level of insulin (A-B) and cortisol (C-D) was determined in individuals with and without diabetes. In A and C Mann Whitney T tests were performed, and in B, D. Kruskal-Walis tests with Dunn's post-hoc test was used: Comparing all pairs of columns. A p-value of < 0.05 was regarded as statically significant.

3.6. Glycaemic control was positively associated with neutrophils and red blood cells, and negatively associated with lymphocytes

The high bacterial burden in PBMCs and MNs of diabetes individuals was associated with glycemic control, we went further to investigate whether there was a relationship between glycemia and other immune cells. We correlated HbA1c and FBG levels with the different immune cells. We saw that in HC, HbA1c positively correlated with neutrophil cell count (p=0.04, r= 0.37) (Figure 8 A). There was a positive correlation between HbA1c and red cell count in the DM2 group (Figure 8 C). This is in conjunction with literature that DM2 patients have higher levels of glycosylated red blood cells than healthy individuals do. Similar to HbA1c in HH, FBG also positively correlated with neutrophil cell count (p= 0.02, r=0.39) (Figure 8 E). Interestingly there was a positive trend between neutrophil count and FBG in DM2 (p=0.066, r= 0.5) group (Figure 8 F). Furthermore, there was a negative correlation between lymphocyte count and HbA1c in the HH group (p= 0.04, r= -0.33) (Figure 8 G).

3.7. Cortisol is negatively correlated with pro-inflammatory cytokine IFN-γ in individuals with diabetes

To assess whether there was an association between cortisol and cytokine response in individuals with DM. Cortisol levels were correlated with absolute IFN-γ concentrations, determined in the QFN ELISA, using spearman analysis. This is particularly important since IFN-γ plays an essential role in clearing Mtb (Kaufmann 2006). We saw that in DM group there was a negative correlation between IFN-γ and cortisol (p=0.032, r=0.52) (Figure 10 A). We then investigated whether this correlation would be stronger in individuals who are at risk (preDM2) or have poorly controlled diabetes, and found there was only a significant negative correlation in healthy controls and individuals with diabetes (Figure 10 B).
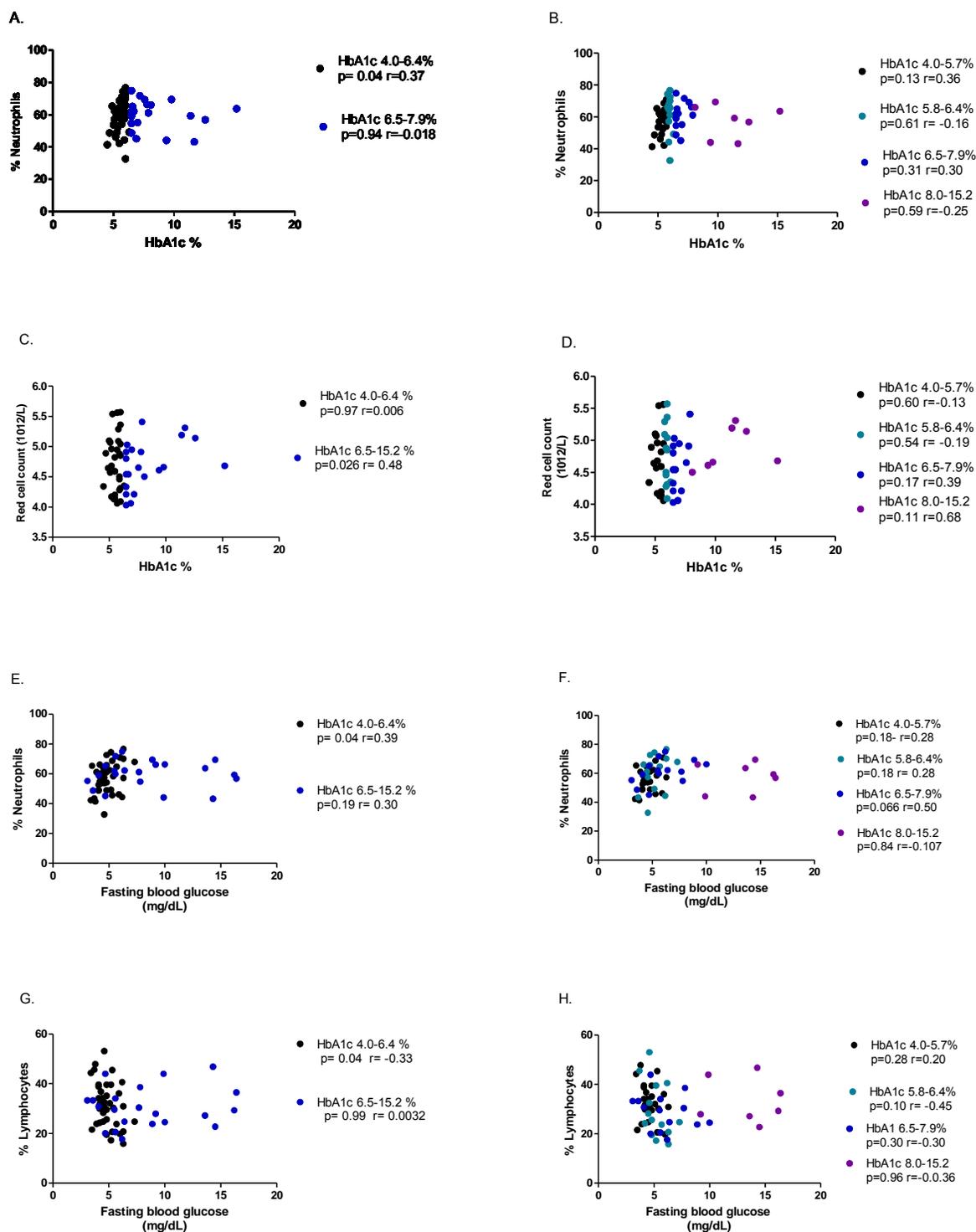
Figure 8. The association between glycemic control and different cell types. Spearman correlations between HbA1c neutrophils and (A-B), red cell counts (C-D) and; between fasting blood glucose and neutrophils (E-F) and lymphocytes (G-H). # Not enough XY pairs to perform statistical analysis.

50
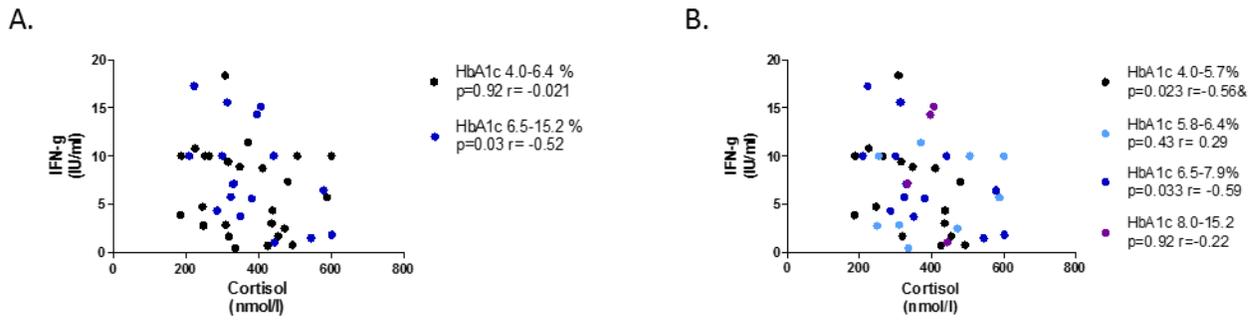
Figure 9. The association between cortisol and IFN-gamma. Spearman correlations between Cortisol and IFN-γ HH vs DM (A) and HC, preDM2, DM2 and pDM2 (B).

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Chapter 4

4. Results study 2

In this chapter, we investigated the immune modulatory properties of cortisol, insulin and leptin, *in vitro,* as these hormones play an important role in DM2 and obesity. We isolated PBMCs and MNs, cultured them with and without hormones, and infected them for 2 hours with Mtb. After the infection, unbound Mtb were washed and the the cells harvested immediately, 24 hours or 72 hours post infection. Cells were lysed and lysates plated to determine CFUs. The parameters for uptake and bacterial burden and/ or killing were the same as study 1. At each time point we collected supernatants to investigate Mtb-induced cytokine production using the luminex platform. In addition, PBMCs were stored and analyzed to determine cell phenotype using flow cytometry.

4.1.   Study participant characteristics

Data from healthy community participants, evaluated in the hormone stimulation assays, are shown in table 3. All participants had a HbA1c of less than 6%. IGRA negative participants were excluded from statistical analysis. There was no significant difference in age between the two groups. There is no BMI information available for the participants.

Table 3. Baseline characteristics of study participants.

|	PBMCs	MNs	P value
N	8	10	
Male (n)	7	6	
Age (years)	37 ± 11	35 ± 13	0,62
HbA1c (%)	5.6 ± 0.48	5.4 ± 0.48	0,40
IGRA positive (n)	8	10	

Values expressed as mean ± SD. Mann-Whitney U t test was performed.

P value <0.05 is significant

4.2.   Optimization of growth inhibition assay and flow cytometry analysis

Before examining the effects of the hormones, we had to take into consideration, which type of media to use. When investigating the effect of hormones, we cannot use autologous plasma to supplement the media, as it contains substances including hormones and glucose. We aimed to exclude these variables from our investigations. Ideally, one would need to use a serum free media such as AIM-V media which contains all the necessary nutrients for optimal growth and activation of differentiated lymphoid cells. An alternative to AIM-V media was to supplement RPMI FBS or Human AB serum however, there are some drawbacks to using either one. The scientific problems encountered with using FBS is that it contains animal-serum components that would induce unexpected cell growth characteristics, cytotoxicity and possible foreign antigen contamination (Tekkatte et al. 2011). An alternative substitute would be to use AB Human serum since it eliminates the secondary effects associated with using FBS, but unfortunately it still contains traces of steroid and peptide hormones (Tekkatte et al. 2011). One way around this problem was to use AB serum with the same lot number to ensure the culture conditions were the same for all cells.

Cells cultured in RPMI supplemented with 10% HI human serum had the highest frequency of live cells in both unstimulated and Mtb stimulated conditions compared to cells cultured in AIM-V media (Figure 11). We therefore used RPMI with 10% HI human serum to culture our cells.
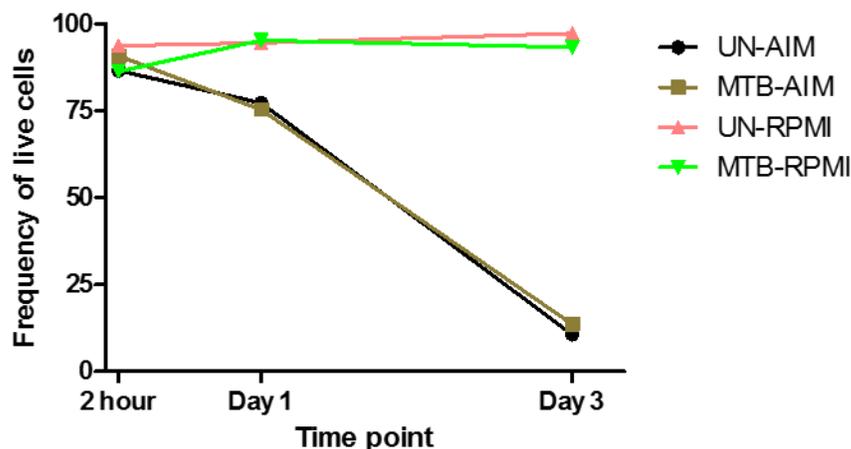

Figure 10. The frequency of live cells cultured in either AIM-V or RPMI supplemented with 10% HI human serum.

UN: Unstimulated, MTB: Mtb stimulated, AIM: AIM-V media, RPMI: RPMI media supplemented with 10% HI human

4.3. The intracellular bacterial control of PBMCs and MNs remain unaffected after hormone stimulations with cortisol, leptin and insulin

We saw that individuals with DM2 have poor bacterial control in PBMCs and MNs, however the causative factor is still unknown. We hypothesized that the poor bacterial control of these immune cells were attributed to a hormonal dysregulation found in individuals with DM2. To test this, we stimulated PBMCs and MNs from healthy community controls with hormones such as cortisol, leptin and insulin at either a low (10^{-9} M) or a high (10^{-7} M) concentration, to investigate whether it would influence the phagocytosis and/ or killing of Mtb. The CFUs were observed at 2 hours, day 1 and day 3 post-infection. We saw that cortisol, leptin or insulin had no effect on the phagocytosis and/ or killing of Mtb in PBMCs, when compared to untreated PBMCs (Figure 11 A). There was a reduction in CFUs on day 1, however this was regardless of the hormone added, or the concentration thereof (Figure 11 A). We then investigated whether these hormones would influence the ability of MNs to control Mtb, since it was previously shown that individuals with DM2 have impaired MN activity. The hormones had no effect on the ability of MNs to control Mtb at the different time points (Figure 11 B). There was an increase in CFUs on day 3, however, this was irrespective of the hormones added, and no dose response was observed (Figure 11 B).
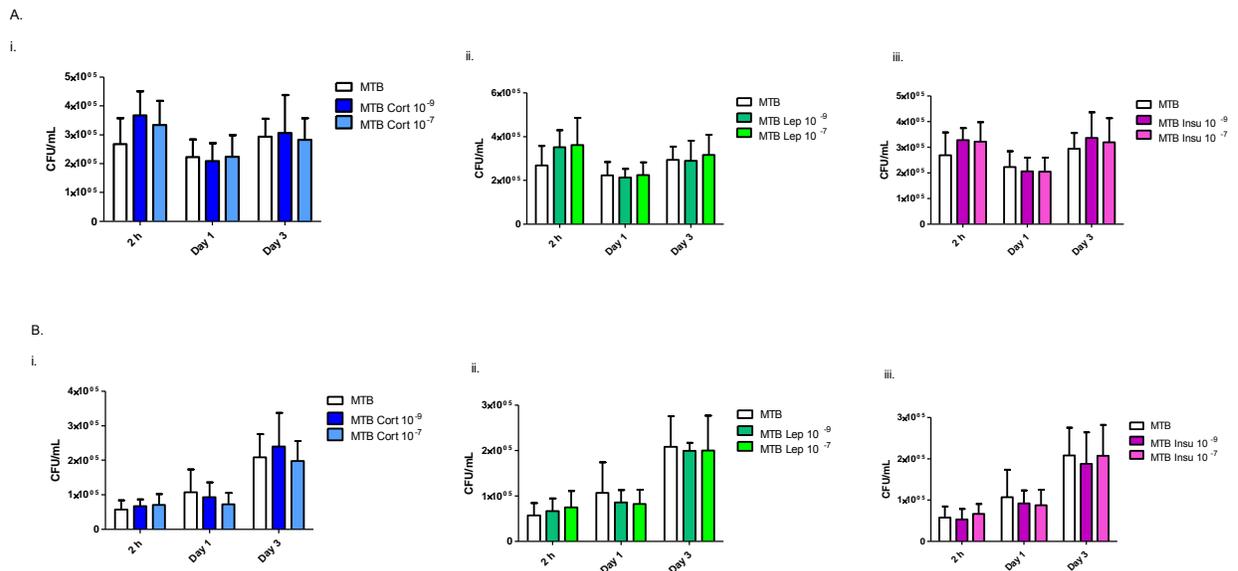
Figure 11. The effect of cortisol, leptin and insulin on the control of Mtb in PBMCs (n=8) and MNs (n=10).

PBMCs (A) or MNs (B) were stimulated with (i) cortisol (blue), (ii) leptin (green) or (iii) insulin (purple) for 16 hours, infected with Mtb for 2h and harvested 2hrs, 1 day and 3 days post-infection. CFUs were enumerated after 3 weeks. A Mann-Whitney U T test pas performed at each time point, by comparing the hormone-treated with untreated group. No statistical significance was observed at each time point. Data represented as mean ± SD.

4.4. Cortisol, leptin and insulin influence cytokine secretion of PBMCs during Mtb infection

Cortisol, leptin and insulin did not alter the phagocytosis and killing of PBMCs infected with Mtb. We therefore investigated whether the hormones influence Mtb-induced cytokine responses that could indirectly contribute to the disease phenotype observed in DM2 patients. To test this, we collected the supernatants of PBMCs that were infected with Mtb and treated with both concentrations (10^{-9} and 10^{-7} M) of cortisol, leptin or insulin. The supernatants were stored at -80°C until analysis. Mtb-induced cytokine concentrations were measured in the supernatant using the Luminex platform. We were limited by cost, therefore were only able to investigate the Mtb-induced cytokine response of PBMCs of 6 individuals and had to pool the supernatants of unstimulated control cultures due to the space and cost constraints of the assay. We only chose the PBMC supernatant of the 6 IGRA positive male participants, since there was only one IGRA positive female participant. From here on the hormone, concentrations will be referred to as high (10^{-7} M) and low (10^{-9} M) concentration.

We determined that the higher concentration of cortisol, 10^{-7} M, influenced cytokine concentrations. At this concentration cortisol significantly decreased IFN-γ production on day 1 and day 3 when compared to the untreated Mtb infected cells (Figure 12 A). Cortisol decreased IL-1b production 2 hours post-infection, whereas on day 3 a significant reduction was observed when compared to the lower concentration of cortisol (10^{-9} M) (Figure 12 B). Cells treated with the higher cortisol concentration produced less IL-6 at 2 hours post- infection, regardless of whether the cells were infected with Mtb however this reduction did not reach statistical significance (p = 0.082). Mtb infection induced IL-6 production on day 1 and day 3 but cortisol had no effect on the production (Figure 12 C). Furthermore, Mtb-induced TNF-α production was significantly decreased by the high concentration of cortisol on day 1 and day 3 (Figure 12 D), together with TNF-β production which was inhibited on day 1 and day 3 (Figure 12 E). IL-8 production was lower in Mtb infected cells treated with the high concentration of cortisol when compared to the untreated, infected cells. (Figure 12 F). Cortisol treatment did not affect Mtb-induced IL-8 responses 1 and 3 days post infection. GM-CSF production was significantly reduced on day 1 and day 3 when cells were treated with the high concentration of the hormone (Figure 12 G).

We continued to investigate whether cortisol influence anti-inflammatory cytokine production of PBMCs and saw that changes in cytokine production was evident with the higher concentration. IL-13 production was significantly reduced by the high concentration of cortisol 1 and 3 days post infection (Figure 13 A). The production of IL-1RA was inhibited by the high cortisol concentration

at all three time points; however, changes observed were similar to uninfected cells, which would suggest that cortisol influenced IL-1RA secretion regardless of the presence of an infection (Figure 13 B). In addition, cortisol decreased the production of IL-5 on day 3 when compared to the other Mtb infected conditions (Figure 13 C). Day 1 post-infection FGF-2 concentrations were higher in the cells treated with a low concentration of cortisol compared to the other two infected groups (Figure 13 D).

Unlike cortisol, the addition of leptin only influenced a small number of cytokines produced by PBMCs. Changes in cytokine concentrations was predominantly observed when leptin was added at a concentration of 10^{-9} M. Mtb infected PBMCs treated with the low concentration of leptin produced significantly less IL-6, 2 hours post-infection. (Figure 14 A). No differences were observed at day 1 and 3. The production of IL-8 was decreased with the addition of high and low leptin concentrations, 2 hours and 1 day post-infection respectively (Figure 14 B). Furthermore, there was a trend that IL-1RA production was decreased at 2 hours (p= 0.082) and day 3 (p= 0.093) in PBMCs infected with Mtb and treated with the lower concentration of leptin, however this was not statistically significantly (Figure 14 C). IL-13 was lower at 2 hours (p=0.053) post-infection (Figure 14 C).

Insulin had no effect on the production of the chemokine VEGF at 2h, day 1 however significantly influenced the production on day 3, at a concentration of 10^{-9} M (Figure 15 A). On day 1, there was a trend that high insulin concentrations induced IL-5 production (p= 0.071) (Figure 15 B). Again on day 3, a trend suggests that IL-1RA production was decreased at both concentrations (insulin 10^{-9} : p= 0.0513 ; insulin 10^{-7} : p= 0.0531). Only TNF-β was increased at 2 hours (insulin 10^{-9} : p= 0.067) (Figure 15 C) and had no effect on the other time points.

The secretion of IL-17A, IFN-α, IL-9 and IP-10 remained unchanged (data not shown).
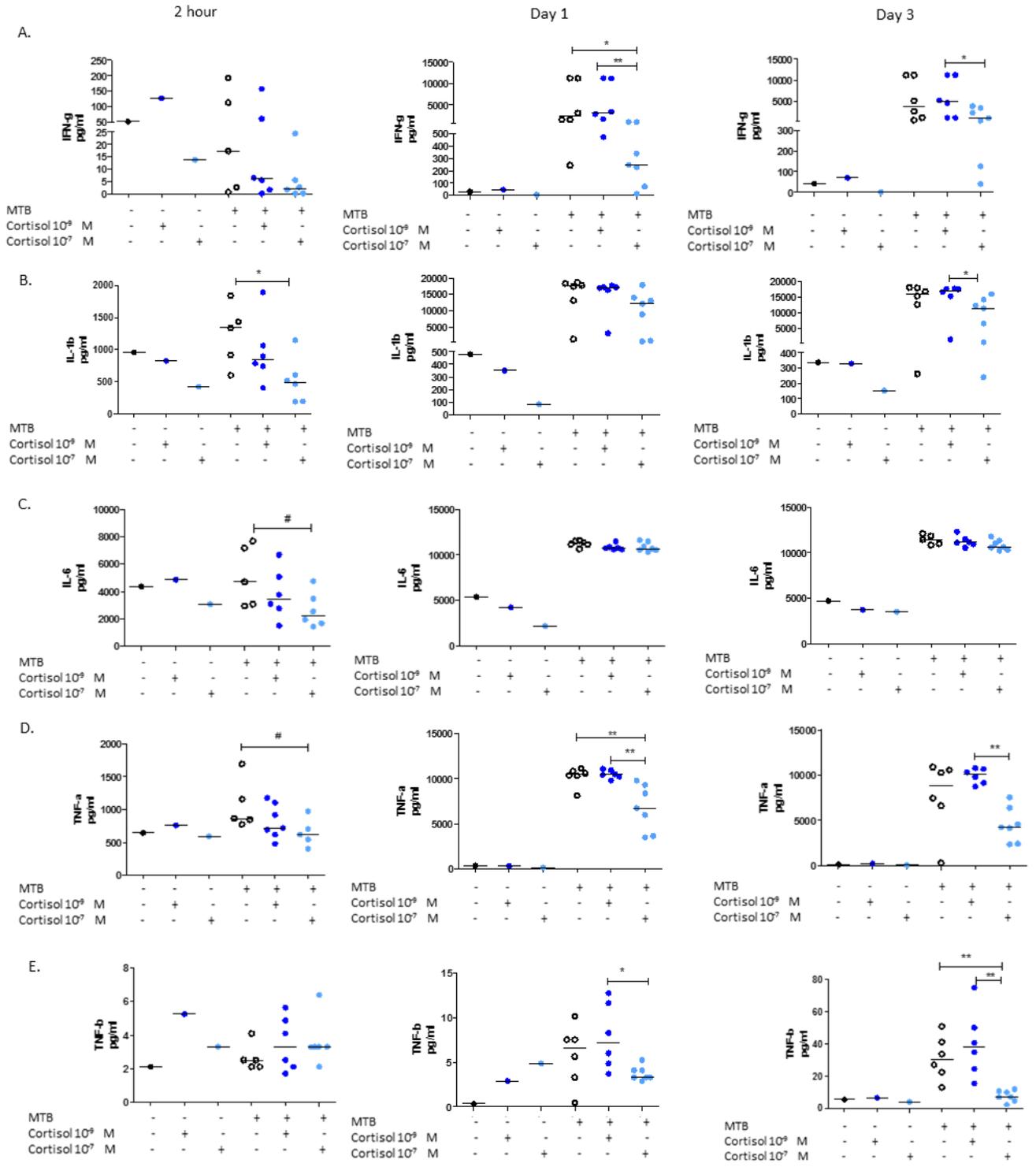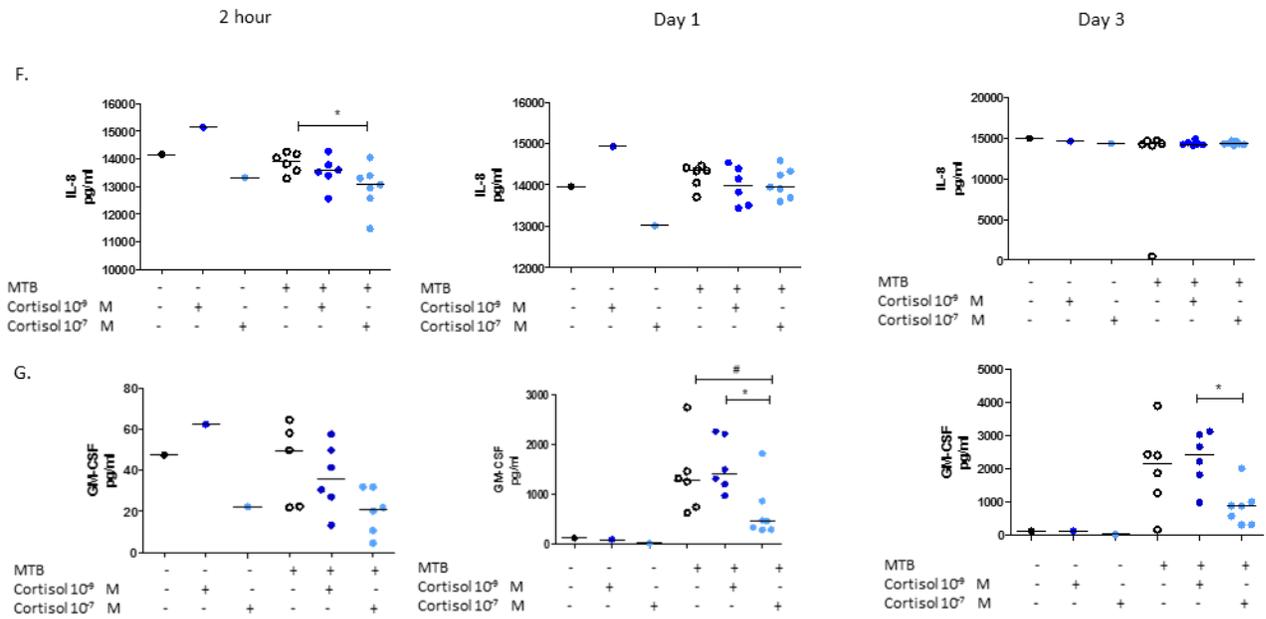
Figure 12. The effect of cortisol on pro-inflammatory cytokine secretion of PBMCs.

The concentration of (A) IFN-γ, (B) IL-1b, (C) IL-6, (D) TNF-α, (E) TNF-β, (F) IL-8 and (G) GM-CSF at 2 hours, day 1 and day 3 were determined by luminex. Data shown for without Mtb is a representative of supernatant pooled from 3 participants, whereas with Mtb infected group one dot represents 1 study participants (n=6). Kruskal-Walis tests with Dunn's post-hoc test was used: Comparing all pairs of columns. Data is presented as mean. ** p < 0.001; * p <0. 01; # p 0.05-0.099
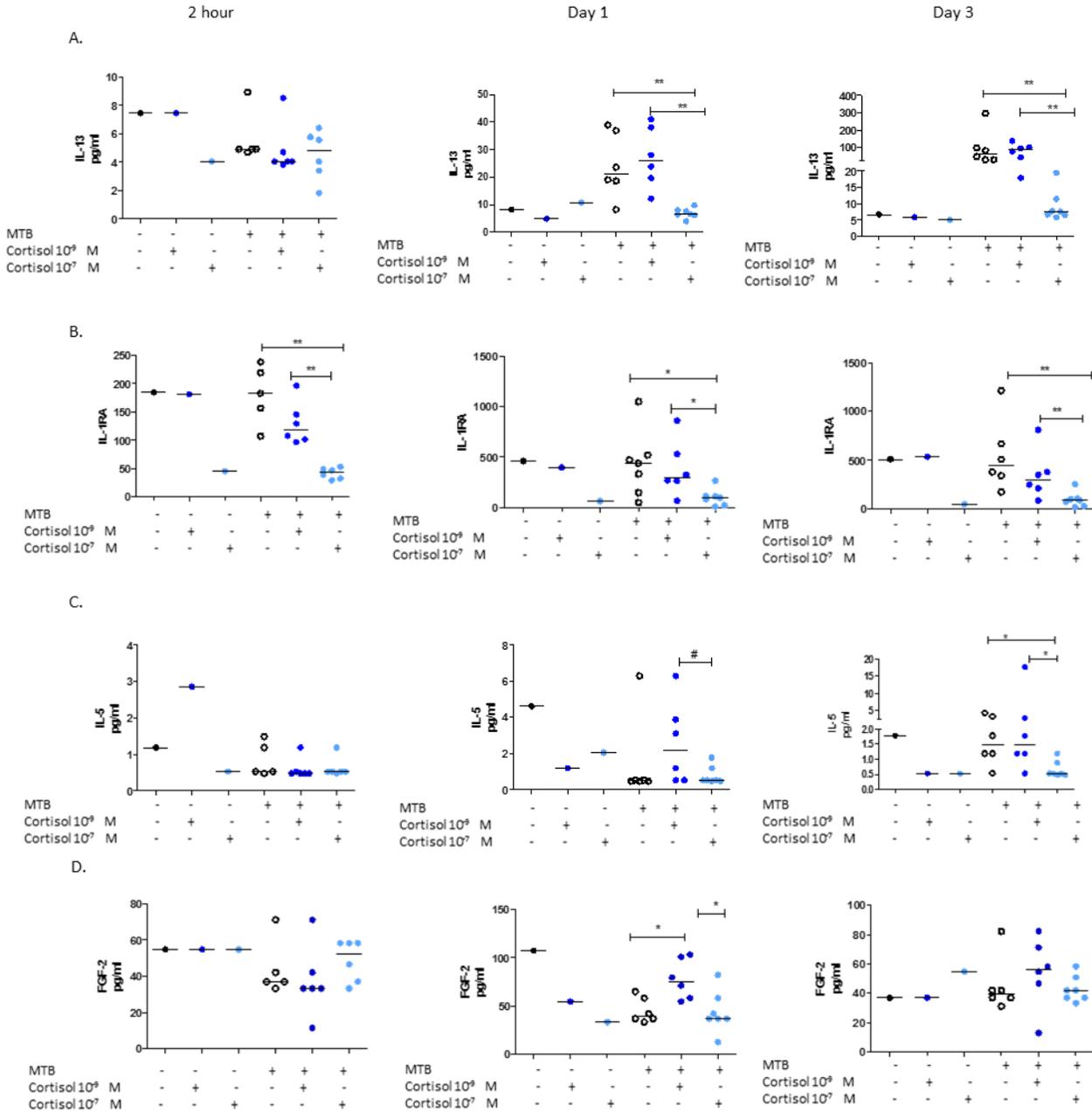
Figure 13. The effect of cortisol on anti-inflammatory cytokine secretion of PBMCs.

The concentration of (A) IL-13, (B) IL-1RA, (C) IL-5 and (D) FGF-2 at 2 hours, day 1 and day 3 were determined by luminex. Data shown for without Mtb is a representative of supernatant pooled from 3 participants, whereas with the Mtb infected group one dot represents 1 study participants (n=6). Kruskal-Walis tests with Dunn's post-hoc test was used: Comparing all pairs of columns. Data represented as the mean. ** p < 0.001; * p <0. 01; # p 0.05-0.099
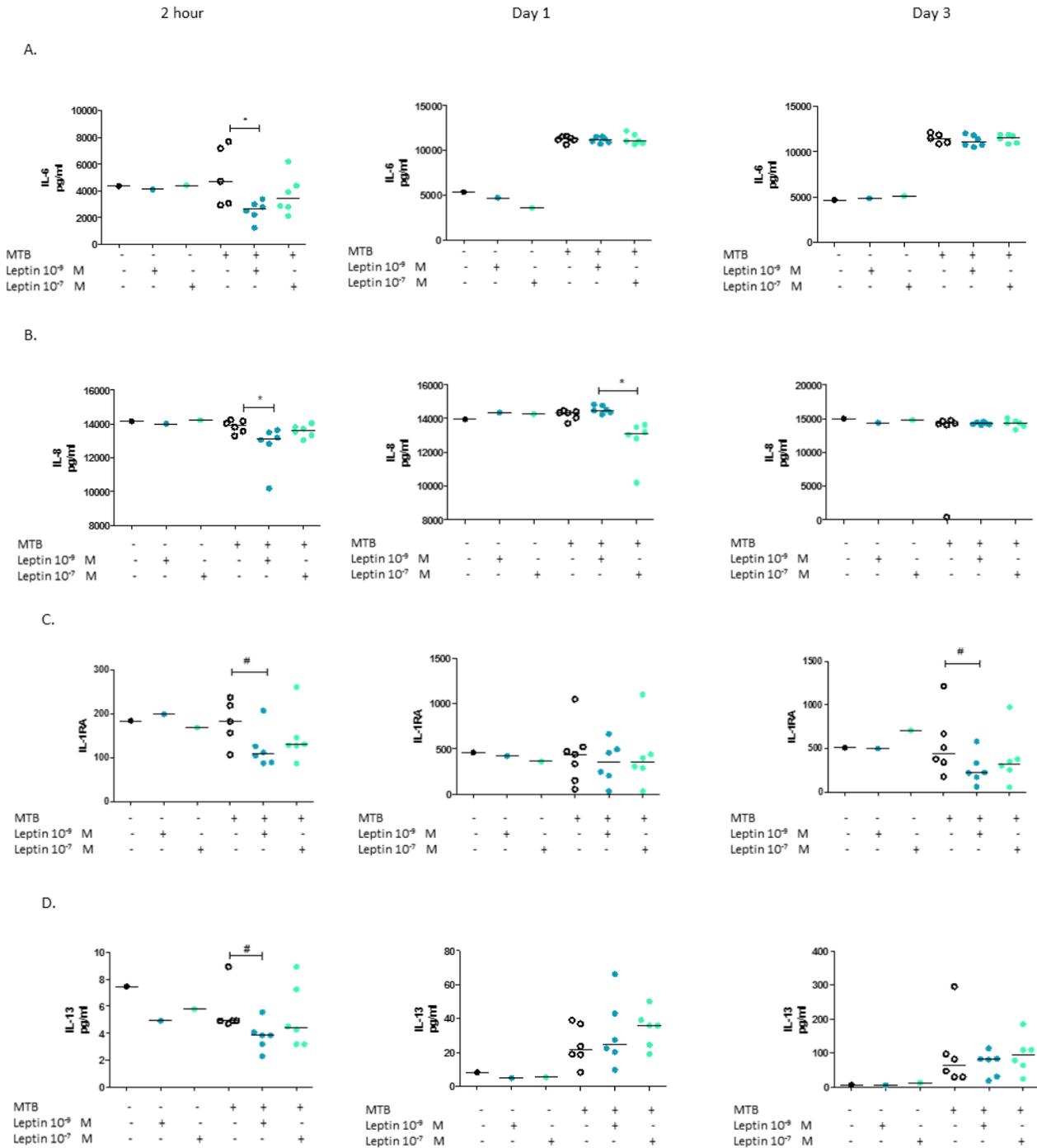
Figure 14. The effect of leptin on pro- and anti-inflammatory cytokine secretion of PBMCs

The concentration of (A) IL-8, (B) IL-1b, (C) IL-1RA and (D) IL-13 at 2 hours, day 1 and day 3 were determined by luminex. Data shown for without Mtb is a representative of supernatant pulled from 3 participants, whereas with Mtb infected group one dot represents 1 study participants (n=6). Kruskal-Walis tests with Dunn's post-hoc test was used: Comparing all pairs of columns. Data represented as the mean. ** p < 0.001; * p <0. 01; # p 0.05-0.099
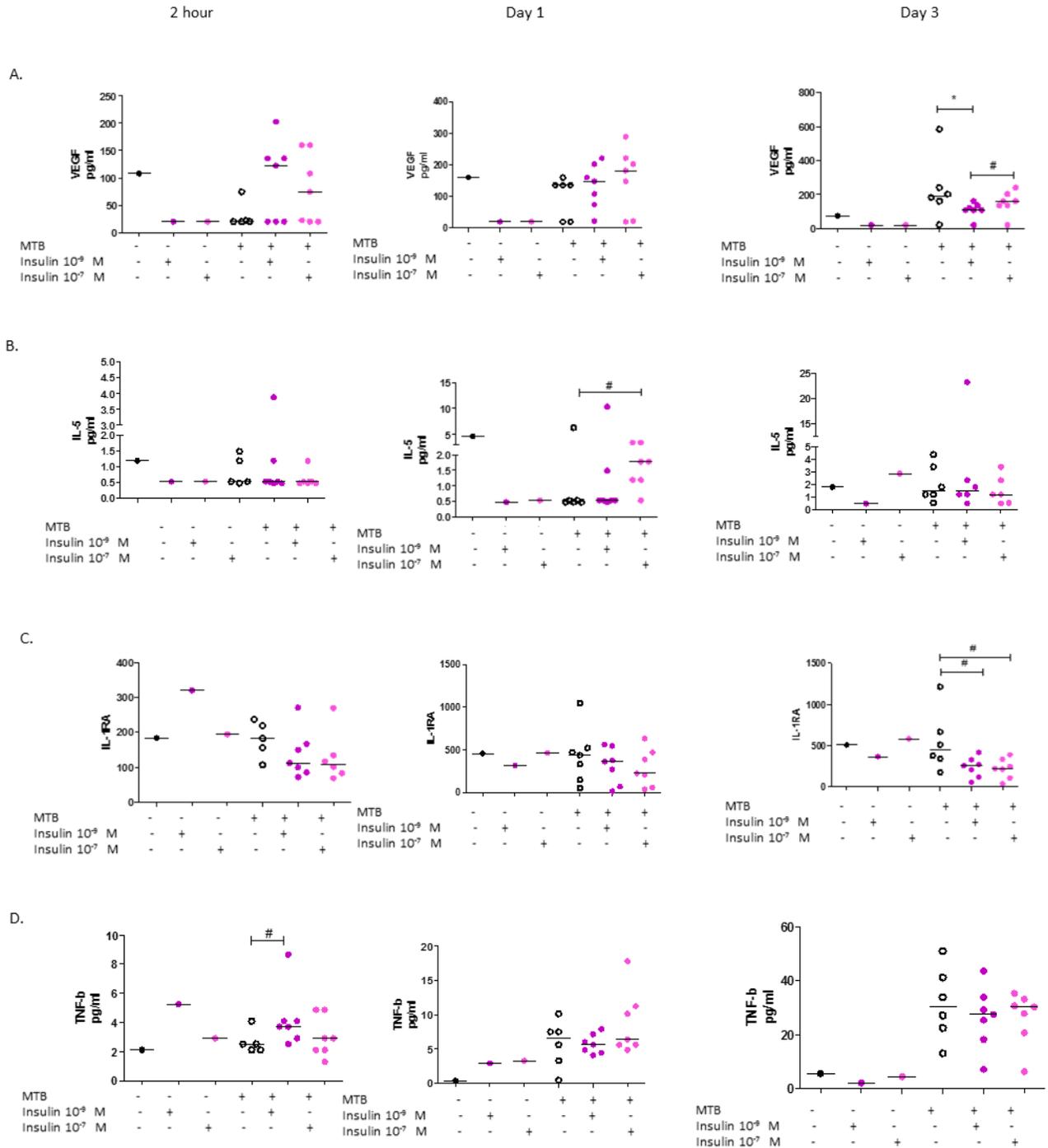
Figure 15. The effect of insulin on pro- and anti-inflammatory cytokine secretion of PBMCs.

The concentration of (A) VEGF, (B) IL-5, (C) IL-1RA and (D) TNF-β at 2 hours, day 1 and day 3 were determined by luminex. Data shown for without Mtb is a representative of supernatant pulled from 3 participants, whereas with Mtb infected group one dot represents 1 study participants (n=6). Kruskal-Walis tests with Dunn's post-hoc test was used: Comparing all pairs of columns. Data represented as the mean. ** p < 0.001; * p <0. 01; # p 0.05-0.099

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4.5. The effect of cortisol, leptin and insulin on the phenotypic characterization of PBMCs during Mtb infection

Both CD4+ and CD8+ T cell populations play an important role in immunity against tuberculosis, and individuals with DM2 are associated with alterations in the number of circulating CD4+ cells (Kumar 2016). We therefore evaluated whether hormones like cortisol, leptin and insulin would alter the frequency of CD4+ and CD8+ T cell subsets during Mtb infection *in vivo*. We examined the percentages of naïve (T_{NAIV}), central memory (T_{CM}), effector memory (T_{EM}) and terminally differentiated effector memory (T_{EMRA}) CD4+ and CD8+ T cells. Figure 16 illustrates the gating strategy used to define these populations. We used the FSC-H vs FSC-A to define our single cells. The SSC-A vs FSC-A defined the lymphocyte population. The SSC-A vs live/dead marker characterized the live cells. From the live cells, we determined our T cell populations. CD3+CD4- defined as CD8+ cells, and CD3+CD4+ was defined as CD4+ cells. From these two populations we determined: CD45+CD197+ T_{NAIV}, CD45+CD197 T_{EMRA}, CD45-CD197- T_{EM} and CD45-CD197+ T_{CM}.

We determined that cortisol at a concentration of 10^{-9} M increased CD4+ T_{EM} population (p= 0.072), however this was not significant, and decreased CD4+T_{CM} (p= 0.053) on day 3 (Figure 18 A). Cortisol had no effect on CD4+ T_{NAIV} and T_{EMRA} cells (Figure 17 A). Leptin however, at a concentration of 10^{-9} M, significantly increased CD4+ T_{EM} and decreased CD4+ T_{CM} populations on day 3 (Figure 17 B). Insulin had no effect on any of the CD4+ populations (Figure 17 C).

When investigating the CD8+ subset populations cortisol and leptin had no effect on the different populations, whereas insulin at a concentration of 10^{-7} M increased T_{NAIV} populations (p= 0.054) (Figure 18 A-C).

As a result of technical difficulties we were unable to detect CD38 and CD127, as a result of low frequencies of live cells. The consequence of this was that CD38 and CD127 were not detected since these markers are expressed in low frequencies.
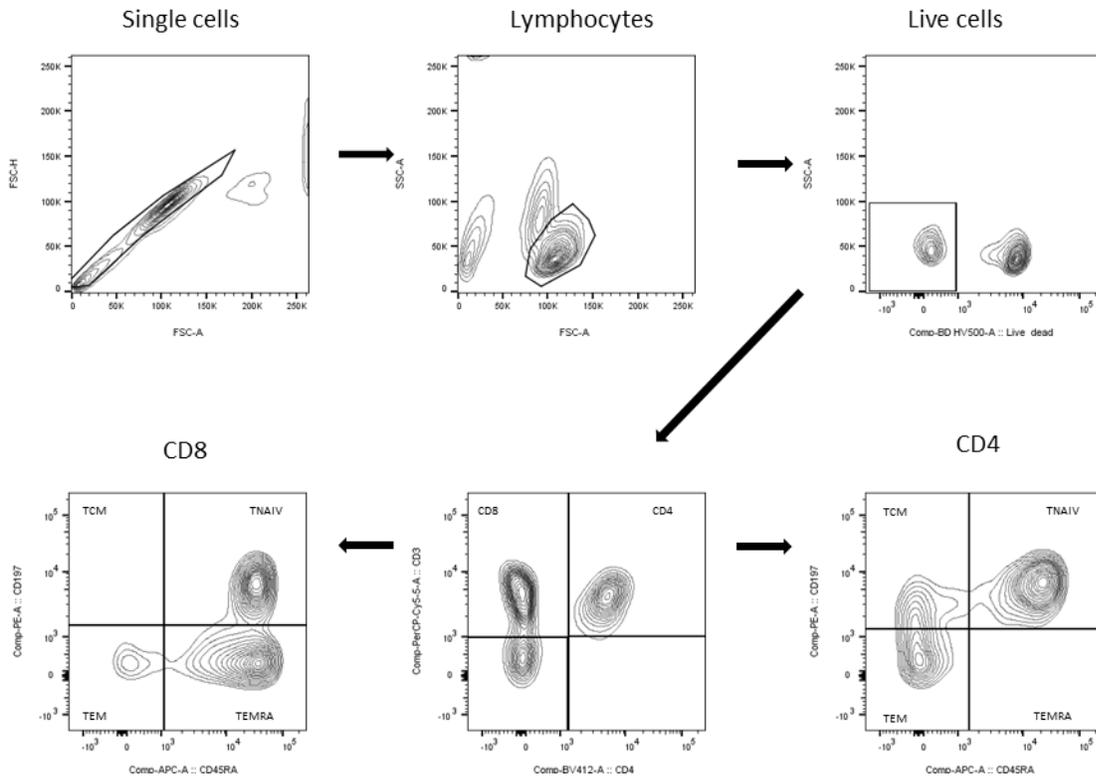
Figure 16. The gating strategy used to define the CD4+ and CD8+ cells.

Cells were acquired using FACS canto II instrument equipped with FACS Diva software, Data was analalyzed using FlowJo software. Gates were determined using florescent minus one (FMO's) and unstained control.

T_{NAIV}: naïve T cells, T_{EMRA}: terminally differentiated effector memory T cells, T_{EM}: Effector memory T cells and T_{CM}: Central Memory T cells.
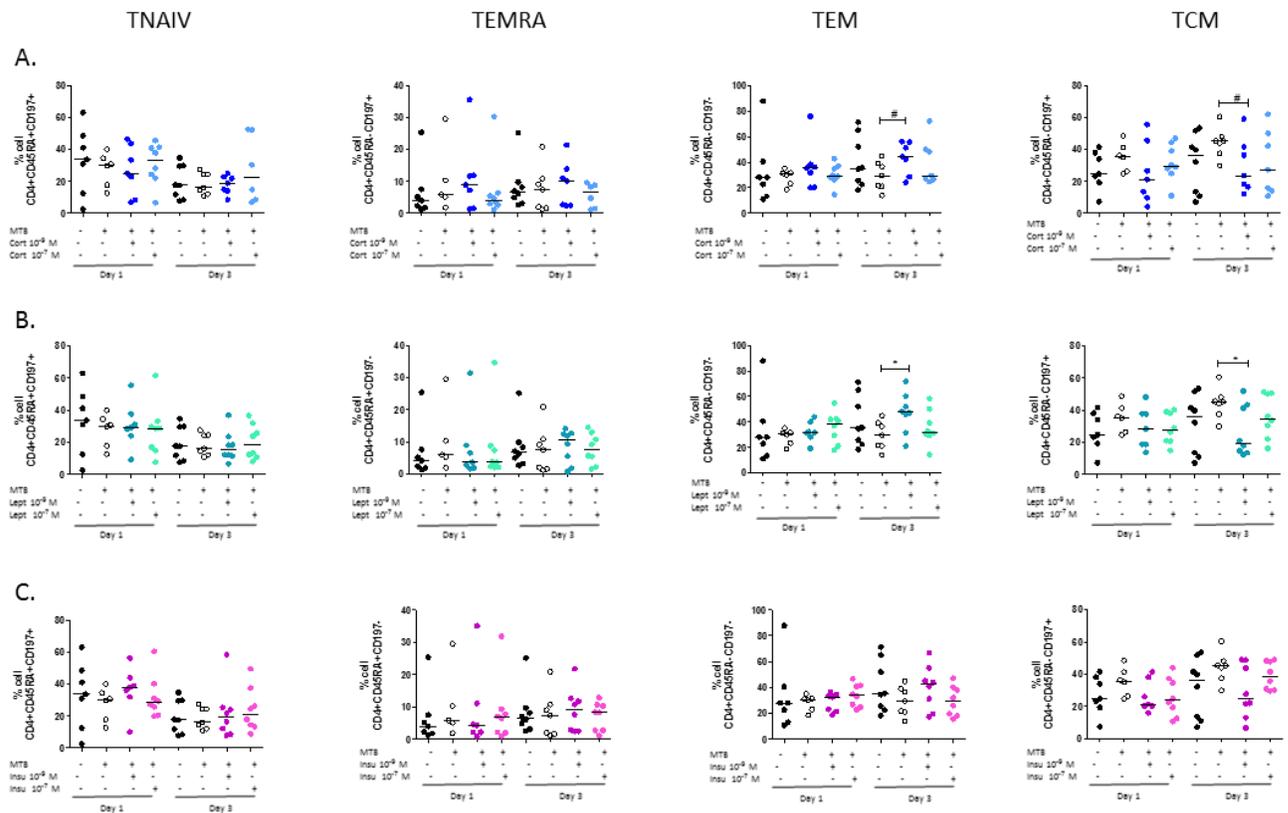
Figure 17. The effect of cortisol, leptin and insulin on CD4+ characterization of PBMCs during Mtb infection.

PBMCs were isolated from healthy donars, and stimulated with (A) cortisol, (B) leptin and (C) insulin for 16 hours. A 2 hour Mtb infection followed, and on day 1 and day 3 cells were collected and stored away at -80 °C. The frequency of the different populations were determined using Flow Cytometry. Each dot represent 1 study participant (n=8). Kruskal-Walis tests with Dunn's post-hoc test was used: Comparing all pairs of columns. At each time point. Data represented as the mean. * p < 0.05; # p 0.05-0.09

Figure 18. The effect of cortisol, leptin and insulin on CD8+ characterization of PBMCs during Mtb infection.

PBMCs were isolated from healthy donars, and stimulated with (A) cortisol, (B) leptin and (C) insulin for 16 hours. A 2 hour Mtb infection followed, and on day 1 and day 3 cells were collected and stored away at -80 °C. The frequency of the different populations were determined using Flow Cytometry. Each dot represent 1 study participant (n=8). Kruskal-Walis tests with Dunn's post-hoc test was used: Comparing all pairs of columns.for each time point. * p < 0.05; # p 0.05-0.09

Chapter 5

Discussion

The Mtb bacterium is capable of surviving and persisting within host mononuclear cells. Macrophages ingest invading bacteria and kill them by different mechanisms including enzymatic generation of reactive oxygen and nitrogen species, and proteolytic digestion (Kaufmann 2006). However, Mtb have strategies for evading intracellular degradation. These microorganisms can thrive in host phagocytes, favoring the development of infectious disease (Kaufmann 2006). In a host with DM2 the bacterium is even more successful, DM2 adults have a 3-fold risk in developing active TB (Jeon and Murray 2008); however, the underlying biological explanation to the amplified susceptibility to TB is not fully understood.

In an attempt to explain the increased susceptibility in DM2, we first investigated whether the *ex vivo* killing of Mtb by PBMCs and MNs of DM2 individuals, which were cultured in media supplemented with autologous plasma, were affected. We observed that in both PBMCs and MNs of DM2 patients had a higher bacterial burden compared to the healthy individuals at day 6. We established that changes in Mtb killing were associated with poor glycemic control. This was evident since the bacterial burden in PBMCs and MNs were higher in individuals HbA1c of > 6.5%, and differences were only observed at the later time points, day 6 and 3 respectively. This would suggest that hyperglycemia has an effect on the killing and not the uptake of the bacterium in PBMCs and MNs. This is oppose to what Restrepo *et al* reported, their team found that hyperglycemia is associated with poor uptake by DM2 individuals (Restrepo et al. 2014). However, their cohort included TB naïve participants whereas we included LTBI individuals. During latency, the bacteria influence host translation in order to restrain host defences thus promoting their survival, which is explained by differential expression in genes responsible for translational control (Lee et al. 2016). This could explain why killing of Mtb is affected in LTBI participants and not in TB naïve participants. All participants had a relatively high BMI, and it did not significantly differ from each other thus obesity cannot account for high bacterial burden observed in DM2 group. However, the healthy participants were younger compared to DM2 group.

An interesting observation in our study was that bacterial burden of MNs of preDM2 individuals remained unaffected throughout all time points, and did not significantly differ from both healthy MNs and diabetic MNs. In a study investing preDM2 in the context with active TB, it was shown

that there was no relationship between HbA1c and bacterial burden (Kumar, Banurekha, et al. 2014). In addition, no relationship between systemic cytokine concentrations and HbA1c was observed. Even though we did not measure cytokine levels, this could account for the lack of changes observed in bacterial burden in our preDM2 group throughout the three time points. This would suggest that there was a balanced network of pro- and anti- inflammatory cytokines, accounting for the lack of differences observed in the bacterial burden. In addition, since the glycemic variables present in preDM2 are higher than normal, but lower than DM2 would indicate that abnormalities caused by hyperglycemia were not detectable. These abnormalities include increased oxidative stress which is associated with chronic or poorly controlled hyperglycemia during DM2, which in turn alters immune response, for example, causing a reduction in IL-12 which causes a reduction in IFN-γ. These results have been correlated with the addition of anti-oxidants (Tan et al. 2012). Furthermore, DM2 patients have a reduction in gene expression for the following genes compared to preDM2 individuals; vacuolar ATPase (ATP6V1H) (Molina et al. 2011), hexokinase 2 (HK2) and CD28 (Qu et al. 2012) which encode for products that contribute to anti-mycobacterial activities of phagocytes. These genes play a role in facilitating Mtb killing and acidifying the phagosome-lysosome compartment, aerobic glycolysis which is the main energy source for macrophages and, co-stimulation of T lymphocytes by antigen-presenting cells respectively (Molina et al. 2011; Qu et al. 2012).

However, the correlation data suggested that there was an association between FBG and CFUs at 2h and day 6, and a trend was observed with HbA1c. This would indicate that there was an association between hyperglycemia and both uptake and killing. This would further support that poor glycemic control as a driving force behind the poor killing and potentially affect uptake of the bacterium by PBMCs. In further support that there was an association between poor glycemic control and bacterial uptake, there was a negative correlation with HbA1c and FBG with CFUs monocytes at 2h of healthy individuals, whereas in DM2 patients there was positively correlation with CFUs and FBG at the same time point. This would suggest that MNs from healthy individuals were able to control bacterial growth better due to less circulating glucose in the blood.

During an Mtb infection, immune cells such as dendritic and monocyte-derived macrophages are primarily involved the initial uptake of Mtb (van Crevel, Ottenhoff, and van der Meer 2002). During the initial encounter Mtb uptake involves different receptors on these immune cells, including complement receptors (CR1, CR3 and CR4), which recognises opsonised Mtb with complement factor C3. Complement factor C3 split to produce C3a which activates the complement system. Mtb binds directly to C2a, which forms C3b which is necessary for binding to CR1 (van Crevel,

Ottenhoff, and van der Meer 2002). However, Mtb uptake can also take place through non-opsonin-mediated phagocytosis in which terminal mannose residues of Mtb is recognised by mannose receptor. When uptake by these two pathways are blocked, macrophages may uptake Mtb through the type A scavenger receptor (van Crevel, Ottenhoff, and van der Meer 2002). Furthermore, other receptors such as Fcγ, aid the uptake of Mtb which are coated by with antibodies of immunoglobulin G class (van Crevel, Ottenhoff, and van der Meer 2002). Once the mycobacterium is phagocytised, the bacterium enters the phagosome, which then undergoes acidification followed by fusion with lysosomes. In the phagosolysome of the activated macrophage the production of reactive oxygen species (ROS) (a super oxide and hydrogen peroxide) or reactive nitrogen species leads to the killing of Mtb. In addition, the activation of macrophages also lead to the production of cytokines which facilitates in the killing of the bacterium. These cytokines are either involved in the initial containment or killing of bacteria (eg. IL-1β and TNF-α) or recruit other immune cells to induce an inflammatory response to aid in the killing of the bacterium (eg. IL-12) (van Crevel, Ottenhoff, and van der Meer 2002).

Most studies suggest that impairment in phagocytic cells is associated with hyperglycemia in DM2. Lower percentage of active phagocytic cells were found in DM2 patients (Lecube et al. 2011). By improving glucose control in DM2 patients, with insulin and metformin treatment, in turn improved phagocytic activity (Lecube et al. 2011). Furthermore, treating monocyte derived macrophages (MDMs) with glucose (16-22mM) reduce phagocytosis of Mtb (Montoya-Rosales et al. 2016). Reduced phagocytosis could be attributed to functional defects via the complement or Fc-γ receptor pathway (Restrepo et al. 2014). However, others showed that high glucose treatment of MNs and macrophages induces M2-type inflammatory phenotype polarization (Torres-Castro et al. 2016) which is counter intuitive since M1-type phenotype is most important for Mtb uptake.

DM2 is considered as a low-grade chronic inflammatory disease, resulting in a dysregulated immune system (Donath and Shoelson 2011). We therefore continued to investigate whether there was a relationship between CFUs observed and the frequencies and absolute numbers other immune cells in the whole blood. We observed that CFUs in PBMCs of HCs at 2h positively correlated with WCC and MN counts. This would suggest that during uptake of Mtb at 2 h in HCs, there was a higher number of monocytes that were able to take up the bacteria, therefore more monocytes available to induce killing at the later time point which resulted in decreased bacterial burden. In DM2 patients, the CFUs of PBMCs at 2h positively correlated with neutrophils. Neutrophils, which are phagocytic cells, are present in the early innate response which could

possibly assist in controlling infection (Sherwood and Toliver-Kinsky 2004). Interestingly, CFUs in MNs of HCs, at 2h, positively correlated with eosinophils and basophil counts whereas in the DM2 group there was a negative trend. This would indicate that in these two groups there was a difference in the interactions and association between these different cell types. In adipose tissue, eosinophils promote M2 polarization and reduce inflammation (Zhang et al. 2015). Eosinophils play a regulatory role in metabolic homeostasis through maintaining adipose M2 macrophages and by secreting Th2 cytokines (Zhang et al. 2015).

In a further attempt to understand chronic inflammation during DM2, we compared different whole blood cell counts between healthy and DM2 patients. Individuals with DM2 had a higher WCC, neutrophils counts (and this was particularly higher in individuals with poorly controlled DM2), borderline lymphocyte counts and decreased MN count. Hyperglycemia leads to alterations in cellular metabolism as a result of ROS and non-enzymatic glycation which results in the formation of advanced glycation end products (AGEs) (Biadgo et al. 2016). AGEs directly activate neutrophils to induce inflammatory cytokines and ROS production. This could explain why there is an elevation of neutrophils in pDM2 (Biadgo et al. 2016). Neutrophils are beneficial during initial containment of Mtb, however, during chronic TB it may delay bacterial clearance (Marzo et al. 2014). Furthermore, HbA1c and FBG positively correlates with neutrophils counts in both healthy participants and DM2 patients.

There is an association between total WCC, neutrophil and lymphocyte counts but not MN counts with DM2 (Gkrania-Klotsas et al. 2010). In contrast to what we found, Nakhajavani *et al.* found that DM2 patients had higher levels of both WCC and MN counts (Nakhjavani et al. 2015). These authors further observed no differences between other cell types such as neutrophil, lymphocyte and eosinophil counts (Nakhjavani et al. 2015). The major differences between the two studies being the sample size (272 DM2 patients vs. 21 DM2), criteria for diagnosing DM2 (HbA1c of 7% vs. 6.5%) and that we included latently infected individuals, whereas Nakhjavani et al did not control for latency. Relative to the incidence of DM2 other studies suggest that leukocyte, neutrophil and lymphocyte were not related to progression of DM2 (Guo et al. 2015). However, we observe differences in lymphocyte counts in our different groups even though there were no differences in the BMI among the groups in study 1. The different groups had a relatively high BMI, and did not significantly differ from each other. In predicting the incidence of DM2, by using HOMA-IR (insulin resistance/sensitivity) and measurement of high-sensitivity CRP (hsCRP) (prediction of subclinical inflammation) models, lymphocyte counts were higher in individuals who develop DM2 and were strongly associated with insulin resistance rather than inflammation

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(Lorenzo, Hanley, and Haffner 2014). Furthermore, neutrophil and monocyte counts were associated with subclinical inflammation and glucose tolerance, whereas WCC was associated with insulin resistance and inversely with insulin secretion (Lorenzo, Hanley, and Haffner 2014). Furthermore, a reduction in MN counts may result in decrease secretion of necessary pro-inflammatory cytokine such as TNF-α, IL-1β and IL-12 which are necessary for the early containment of Mtb (van Crevel, Ottenhoff, and van der Meer 2002). We therefore can deduce that multiple mechanisms may contribute to inflammation in DM2 such as elevation of certain cell types which was either induced by insulin resistance, glucose intolerance or subclinical inflammation. Since most of these studies were investigating pathogenesis of DM2 or predicting the incidence of DM2 one can speculate that the inflammation observed in DM2 is a cause of increased susceptibility to bacterial infections. The different mechanisms inducing inflammation may collectively or independently contribute to susceptibility to bacterial infections such as Mtb. Therefore, a proof-of-concept study should be done to investigate whether the dysregulation of immune cells is a causative factor for the increased susceptibility to Mtb infections.

Even though DM2 individuals have higher WCC, they had lower percentage of MNs. The low percentage of MN observed in DM2 individuals could be a result of the migration of MNs to other tissues eg. adipose tissue, lungs or lymph nodes (since the participants are latently infected). Both pro-inflammatory and metabolic stimuli increase the recruitment of monocytes to peripheral tissues, where they differentiate to macrophages and dendritic cells (Sherwood and Toliver-Kinsky 2004). This would indicate that the destination of monocytes is therefore not the bloodstream. Thus, the periphery does not give a true presentation of monocytes presence or give an indication of monocyte-mediated tissue effect. In addition, it has been reported that there is a difference in the expression of chemokine receptors (e.g. CCR2 marker on MNs) in blood between TB-DM2 and DM2 (Stew et al. 2013), which further supports that there are differences in cell numbers at different location.

In this study DM2 individuals had higher levels of insulin, which is in agreement with the literature. Due to the high insulin levels it is possible these individuals had insulin resistance or decreased insulin sensitivity, as an account to the high glucose in the blood. Interestingly, cortisol levels remained unchanged through the different groups. Since our DM2 cohort does not have any major health complications, except being latently infected with Mtb, it suggests that latent Mtb infection does not have a large enough impact to influence cortisol levels. Cortisol levels are elevated depending on the severity of the complications accompanying DM2 (Chiodini et al. 2007) and during active TB (Natalia Santucci et al. 2011a).

Despite the fact that cortisol levels remained unchanged in the different groups, cortisol levels in DM2 patients negatively correlated with IFN-γ concentrations. Suggesting that cortisol, in DM2 patients, has immune modulatory effects by down regulating Th1 response.

Since literature suggest that hormones such as cortisol, leptin and insulin are dysregulated in DM2 individuals, it was likely that these hormones would modify the immune responses of PBMCs and MNs to Mtb infection, by suppressing or down regulating Th1 immune response or favour a M2 phenotype. These responses could either directly or indirectly contribute to the disease phenotype observed in DM2 patients.

Cortisol is a glucocorticoid hormone secreted by the adrenal gland and plays a role in the stress response. We found that cortisol did not influence the bacterial uptake or killing in both PBMCs and MNs. However, we observed that cortisol has an anti-inflammatory effect given the decreased production of the pro-inflammatory cytokines IL-1β, IL-6, TNF-α, TNF- β, IL-8, IFN-γ and GM-CSF. TNF-α, IL-1β, IL-6, GM-CSF are cytokines secreted by macrophages that act as mediators during the acute inflammatory response to bacteria (Sherwood and Toliver-Kinsky 2004). Furthermore, IL-1β and TNF-α induce IFN-γ production by Th1 cells (Sherwood and Toliver-Kinsky 2004), and this would suggest the decreased production of these cytokines led to the reduced IFN-γ response on day 1 and day 3. This is in conjunction to what Bongiovanni *et. al* found that cortisol did not have an effect on the intracellular Mtb control, but decreased the phagocytosis of Mtb (Bongiovanni et al. 2012). Not only was the phagocytosis inhibited, but also the production of TNF-α, IL-1β and IL-10 (Bongiovanni et al. 2015). Cortisol treatment results in the inhibition of proliferation of PBMCs and a reduction in Mtb induced IFN-γ responses in HHCs (Mahuad et al. 2004). This response was even more profound in individuals with active TB (Mahuad et al. 2004). Collectively, the data suggest that the concentration of cortisol within physiological range can inhibit immune responses to mycobacterial antigens during TB. Th1 activity, particularly IFN-γ, plays an important role in bacterial clearance during Mtb infection (O'Garra et al. 2013). This coincides with our data that there is a negative correlation between IFN-γ and cortisol in DM2 patients. IFN-γ is important in activating macrophages for the killing of intracellular mycobacteria (Kaufmann 2006). Furthermore, the reduction in IL-8 production, a chemokine responsible for cell recruitment, could potentially lead to decrease recruitment of neutrophils to site of infection (Sherwood and Toliver-Kinsky 2004) which in turn could lead to reduction in bacterial control. Together this would suggest that cortisol has anti-inflammatory properties by suppressing Th1 response through supressing IFN-γ as well as other pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, GM-CSF). Furthermore, since DM2 patients have

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elevated neutrophils, the reduction in IL-8 induced by cortisol could potentially limit recruitment of DM2 neutrophils from the blood to the site of infection.

Leptin is an adipokine that is secreted primarily by adipose tissue (Grant and Dixit 2015). Similar to cortisol, leptin did not have an effect on the bacterial killing in both PBMCs and MNs. With regards to the Mtb induced cytokine response of PBMCs, leptin decreasing the production of the Th2 cytokines IL-13 and IL-5 induces a Th1 response. Leptin is known to stimulate the production of IL-1β (Gorlé et al. 2016), however in our study leptin had no effect on IL-1β production. This was observed even though IL-1RA, which is a IL-1β receptor antagonist, production was decreased. There are discrepancies in the production of IL-1RA and IL-1β induced by leptin treatment in different studies. Leptin treatment in an acute monocytic leukemica cell line (THP-1 cell line) and PBMCs induces the production of IL-1RA (Gabay et al. 2001), whereas a more recent study showed that mycobacterium induced cytokine production of IL-1β and IL-1RA, in THP-1 cells, was not influenced by leptin treatment (N. Santucci et al. 2014). Differences between the studies can be attributed to the different stimulation methods used (phytohaemagglutinin (PHA) versus Mtb antigens). Another key cytokine, TNF-α, that leptin has an association with, was unaffected. Leptin treatment induces and activates the proliferation of macrophages and monocytes and stimulates the production of IL-6 and TNF-α (Sánchez-Margalet et al. 2003). Furthermore, macrophages treated with the hormone express surface markers that resemble a M2-phenotype, but were able to produce M1-type cytokine production such TNF-α, IL-6 and IL-1β (Acedo et al. 2013). In addition, some studies suggest that leptin on its own does not induce TNF-α production in human MNs (Terán-Cabanillas and Hernández 2016). Upon mycobacterial stimulation, leptin diminished the proliferative ability of PBMCs and had no effect on IFN-γ production in both supernatant culture and intracytoplasmic analysis (N. Santucci et al. 2014). In studies, investigating supra-physiological treatment of leptin, during PHA stimulation, increases the proliferation of PBMCs and lymphocytes and the production of IFN-γ (Martín-Romero et al. 2000). Together the data suggest that leptin does not increase production of IL-1β, which therefore could not potentially activate the hypothalamus which in turn would result in the increase production of cortisol. Instead, it may suggest that leptin induces a Th1 response by decreasing Th2 cytokines, however it does not improve bacterial control as indicated with CFU data in PBMCs and MNs.

Insulin, the pancreatic hormone, did not influence the bacterial burden in both PBMCs and MNs which was a similar observation seen as with cortisol and leptin. To our knowledge, this is the first study that investigate the effect of insulin treatment on PBMCs and MNs in the context Mtb

infection. Insulin decreased the production of IL-1RA, a receptor antagonist, which could lead to increased inflammation in DM2. IL-1RA is used as corrective measure to improve beta cell dysfunction. When an imbalance between IL-1β and IL-1RA occurs, with a higher IL-1β compared to IL-1RA, it leads to pancreatic islet inflammation and increased insulin secretion, which could contribute to the increased low-grade systemic inflammation in DM2 if the imbalance persist (Akash et al. 2012). When lymphocytes are exposed to supraphysiologic levels of insulin it can induce the Th2 polarization of these cells (Dube et al. 2015), which we observed with the increase production in Th2 cytokine IL-5. As mentioned above, a Th1 immune response is needed for bacterial clearance. Insulin further decreased the production of VEGF, secreted by Mtb-infected macrophages, which could potentially be beneficial for the host. It has been shown that inhibition of VEGF results in a reduction in the bacterial burden in the lungs, spleen and lymph nodes in mice infected with Mtb (Polena et al. 2016). The bacillus may exploit VEGF-mediated angiogenesis to increase its dissemination or continue to reside inside the host (Polena 2016). Therefore, a reduction in VEGF may mean that the spread of the bacillus in a host will be limited. In addition, VEGF is involved in the pathogenesis of DM2, and contribute towards the vascular complications associated with DM2 (L. Guo et al. 2014).

There were limitations in the study, including small sample size used. Furthermore, the data analysis did not adjust for confounding factors associated with DM2. Even though we were unable to detect any changes in bacterial burden in the hormone treatment *in vitro* study, the changes in Mtb induced cytokine production does show that cortisol, leptin and insulin alter immune responses. The cytokine production should therefore be validated in a bigger cohort. Furthermore, our cohort mainly consisted of IGRA positive males; it would be beneficial to broaden the cohort to include equal numbers of males and females. Due to cost restraint, we were limited to space in the luminex analysis and were only able to include one set untreated and unstimulated sample type. In addition, improper storage of PBMCs resulted that we did not detected any differences in T cell phenotypes, and unable to determine all the target subpopulations due to low cell viability.

Conclusion

In DM2, phagocytosis and killing efficacy of PBMCs and MNs from CC of TB patients were associated with physiological changes characteristic to DM2. Poor bacterial control in DM2 associated with hyperglycemia, chronic inflammation induced by increased WCC, neutrophil, and lymphocyte counts. The level of cortisol in DM2 individuals negatively correlated with IFN-γ, thus

suppressing Th1 response. Furthermore, the study indicate that endogenous hormones such as cortisol, leptin and insulin could mediate some cytokine response in DM2 patients. Cortisol suppress macrophage activation or Th1 activity, which could lead to poor bacterial control. Whereas, leptin upregulates Th1 response that may improve bacterial control. However, its role still remain undetermined. The role of insulin is debatable as it may either induce a Th2 response, which could lead to poor bacterial control, or may a play a role in preventing the spread of Mtb to other organs by decreasing the production of VGEF. Therefore, during DM2, immune alterations are associated with decreased bacterial control, and that endocrine factors such as cortisol would suppress Th1 response, regardless whether it elevated or not. Thus, would potentially exacerbate bacterial control in cases where DM2 is worsened by other complications.

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