Identification of distinct bio-signatures in whole blood and in-tube Quantiferon pellets of Tuberculosis (TB) close contacts (CCs) and TB patients with and without type 2 diabetes (T2D)

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Abstract

Tuberculosis (TB) remains difficult to control despite effective treatment and the presence of more sensitive diagnostic tools. Immunity against *Mycobacterium tuberculosis* (Mtb) infection can further be impacted by other concomitant infections (like human immunodeficiency virus) and non-communicable diseases (like Type 2 diabetes (T2D)) rendering individuals susceptible to TB. T2D patients are three times more likely to progress from latent to active TB. Therefore, it is crucial to better understand why people with T2D are at increased risk for TB progression. We hypothesize that distinct bio-signatures exist in whole blood of TB patients and close contacts (CCs) of TB patients with latent TB infection (LTBI) with T2D compared to those without T2D and that these bio-signatures correlate with impaired immune responses to Mtb in peripheral blood mononuclear cells (PBMCs) and monocytes (MNs) in CCs.

RNA was extracted from whole blood stimulated with Mtb antigens using the Quantiferon TB Gold in-tube assay and NanoString analysis was performed to determine whether specific transcription signatures exist between CCs with LTBI with and without T2D. We characterized serum cytokines and endocrine signatures by luminex and enzyme-linked immunosorbent assay. PBMCs and MNs from the same individuals were isolated and stimulated with live *H37Rv* Mtb to determine Mtb uptake and killing. Bacterial uptake and killing was correlated with HbA1c to determine whether there was an association with glycaemic control. RNA from TB patients, TB patients with transient hyperglycaemia (TB-THG) and TB patients with T2D (TB-T2D) was also extracted from whole blood stimulated with Mtb antigens and analysed using NanoString. Interleukin-22 was also measured in serum from these patients. PBMCs from TB patients were further used to determine the frequency of mucosal associated invariant T (MAIT) cells before, during and after TB treatment.

Fifteen gene transcripts were downregulated in TB-T2D compared to TB and TB-THG patients. We identified a gene signature that was able to differentiate between TB-THG and TB-T2D. We further identified a gene signature unique to the CCs with LTBI and T2D, which could be associated with an increased risk of developing active TB. Differences in cytokines, hormones, lipids, differential blood counts and Mtb uptake was observed when comparing CCs with and without T2D. Differential expression of IL-6, IL-18 and IL-22 in CCs with and without T2D highlights the differential regulation of the immune response during T2D. We showed that the frequency of MAIT cells in the periphery of TB-T2D was significantly lower compared to TB-THG at baseline, suggesting the MAIT cells in TB-T2D have redistributed to either the lung or adipose tissue. The increase of MAIT cells in the periphery at month 2 compared to baseline could mean that MAIT cells population was restored in the blood due to TB treatment.

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Our gene expression results suggests that downregulated gene transcripts may be involved in pathways that favour immunopathology in TB-T2D. In addition, gene signatures differentiating THG from T2D could be useful in preventing the unnecessary diagnosis of patients with THG as having T2D. Differential gene expression in CCs with LTBI with and without T2D shows potential to be involved in the mechanisms leading to susceptibility to active TB. Cytokine and hormone data confirms that during T2D, the immune response is differentially regulated which may influence the response to infections. Lastly, MAIT cell frequency could be useful for monitoring treatment responses in TB-T2D patients.

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Abstrak

Tuberkulose (TB) bly moeilik om te beheer ten spyte van effektiewe behandeling en die teenwoordigheid van meer sensitiewe diagnostiese toetse. Immuniteit teen *Mycobacterium tuberkulose* (Mtb) kan verder beïnvloed word deur ander gepaardgaande infeksies (soos menslike immuniteitsgebrekvirus) en nie-oordraagbare siektes (soos tipe 2 diabetes (T2D)) wat individue vatbaar maak vir TB. Pasiënte met T2D het ’n groter kans om van latent na aktiewe TB te vorder en is dit noodsaaklik om te verstaan waarom mense met T2D ‘n groter risiko vir TB-progressie het. Ons veronderstel dat daar afsonderlike bio-handtekeninge in heel bloed van TB pasiënte asook nabye kontakte (CCs) van TB pasiënte met latente TB-infeksie (LTBI) en T2D in vergelyking met dié sonder T2D bestaan en dat hierdie bio-handtekeninge met verswakte immuunresponse teenoor Mtb in mononukleêr selle in perifere bloed (PBMCs) en monosiete (MNs) van CCs korreleer.

RNS is uit volbloed wat met Mtb-antigene (Quantiferon TB Gold toets) gestimuleer is onttrek. Nanostring-analise is uitgevoer om vas te stel of spesifieke transkripsie-patrone tussen CCs met LTBI en met of sonder T2D bestaan. Ons het serum sitokiene en endokriene handtekeninge identifiseer deur luminex en ensien-gekoppelde Immunosorbent-toetse. PBMCs en MNs van dieselfde individue is geïsoleer en met lewendige *H37Rv* Mtb gestimuleer om Mtb opname en beheer te bepaal. Bakteriese opname en dood was met HbA1c gekorreleer om vas te stel of daar 'n verband met bloedsuiker was. RNS van TB-pasiënte, TB-pasiënte met oorgang hiperglukemie (TB-THG) en TB-pasiënte met T2D (TB-T2D) was ook uit heelbloed wat met Mtb-antigene gestimuleers was onttrek en met behulp van die Nanostring tegnologie geanaliseer. IL-22 was ook in the serum van hierdie pasiënte gemeet. PBMCs van TB pasiënte is verder gebruik om die frekwensie van ‘mucosal associated invariant’ T (MAIT) selle voor, tydens en na TB behandeling te bepaal.

Vyftien geen-transkripsies is in TB-T2D afwaarts gereguleer in vergelyking met TB- en TB-THG pasiënte. Ons het 'n geen-handtekening geïdentifiseer wat in staat was om tussen TB-THG en TB-T2D te onderskei. Ons het verder 'n handtekening van gene geïdentifiseer wat uniek is aan die CCs met LTBI en T2D wat met 'n verhoogde risiko om aktiewe TB te ontwikkeling geassosieer kan word. Verskille in sitokiene, hormone, lipiede, differensiële bloedtellings en Mtb opname is waargeneem tydens die vergelyking van CC's met en sonder T2D. Differensiële uitdrukking van IL-6, IL-18 en IL-22 in CCs met LTBI met en sonder T2D beklemtoon die differensiële regulering van die immuunrespons tydens T2D. Ons het getoon dat die frekwensie van MAIT selle in die sirkulasie van TB-T2D laer was as TB-THG by basislyn, wat daarop dui dat die MAIT-selle in TB-T2D na die long- of vetweefsel migreer. Die toename van MAIT selle in die bloed by maand 2 in vergelyking met basislyn kan beteken dat MAIT selpopulasies in die bloed herstel, as gevolg van TB behandeling.

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Ons geen-uitdrukking resultate dui daarop dat af-gereguleerde geen-transkripsies betrokke kan wees in immuunpaaie wat patologie in TB-T2D bevoordeel. Daarbenewens kan geen-handtekeninge wat THG van T2D onderskei, nuttig wees om ’n misdiagnose van THG as T2D te vermy. Differensiële geen-uitdrukking by individue met LTBI met en sonder T2D toon potensiaal om betrokke te wees by die meganismes wat lei tot vatbaarheid vir aktiewe TB. Sitokien- en hormoon data bevestig dat tydens T2D, die immuunrespons differensieel gereguleer word, wat die respons op infeksies kan beïnvloed. Laastens, MAIT-sel frekwensies kan nuttig wees vir die monitering van behandelings uitkomste in TB-T2D-pasiënte.

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Abbreviations

Activator protein-1	AP-1
Adrenocorticotropic hormone	ACTH
Antigen	Ag
Antigen presenting cells	APC
Alveolar macrophage	AM
American Diabetes Association	ADA
Broncho alveolar lavage fluid	BAL-F
Bacillus Calmette-Guerin	BCG
Baseline	BL
Bone marrow derived macrophages	BMDM
C-C motif chemokine ligand	CCL
C-reactive protein	CRP
C-type lectin receptors	CLRs
C-X-C motif chemokine ligand	CXCL
Catecholamines	CA
Central nervous system	CNS
Cetyltrimethyl ammonium bromide	CTAB
Chest X-ray	CXR
Clofazimine	Cfz
Close contacts	CCs
Cluster of differentiation	CD
Complete blood count	CBC
Computerised tomography	CT
Corticotropin-releasing hormone	CRH
Culture filtrate protein-10	CFP-10
Dendritic cells	DCs
Dehydroepiandrosterone	DHEA
Diabetes	DM
Dipeptidyl peptidase IV	DPP4
Directly observed treatment short course	DOTS
Early secretory antigen-6	ESAT-6
End stage renal disease	ESRD
Enzyme linked immunosorbent assay	ELISA
Ethambutol	EMB

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Ethylenediaminetetraacetic acid	EDTA
Extensively drug resistant	XDR-TB
Fasting blood glucose	FBG
Follicle-stimulating hormone	FSH
Food and drug administration	FDA
Glucagon like peptide–1	GLP-1
Glucose-6-phosphate dehydrogenase	G6PD
Glucocorticoids	GCs
Glutamine	GLU
Glycated haemoglobin	HbA1c
Granulocyte colony stimulating factor	G-CSF
Granulocyte macrophage colony stimulating factor	GM-CSF
Growth hormone	GH
Healthy controls	HCs
Horseradish Peroxidase	HRP
Host directed therapy	HDT
Human immunodeficiency virus	HIV
Human serum albumin	SEH
Hypothalamic pituitary adrenal	HPA
Impaired fasting glucose	IFG
Impaired glucose tolerance	IGT
Interferon chemoattractant	I-TAC
Interferon-gamma	IFN-y
Interferon-gamma release assay	IGRA
Interleukin	IL
Inter quartile range	IQR
Isoniazid	INH
Janus kinase/signal transducers and activators of transcription	JAK-STAT
Latent Mycobacterium tuberculosis infection	LTBI
Latent Mycobacterium tuberculosis infection with	LTBI-T2D
Type 2 diabetes	T2D
Lipopolysaccharide	LPS
Lipopolysaccharide inducible C-C related gene	L-CCR
Lithium heparinized	Lihep
Luteinizing hormone	LH

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Major histocompatibility	MHC
Major histocompatibility related protein-1	MR-1
Monocytes	MNs
Month 6	M6
Moxifloxacin	Mfx
Mucosal associated invariant T	MAIT
Multi-drug TB	MDR-TB
Mycobacteria growth indicator tube	MGIT
Mycobacterium tuberculosis	Mtb
Natural killer	NK
Natural killer T	NKT
Neutrophil extracellular traps	NETs
Nod like receptors	NOD
Nuclear Factor kappa-light-chain-enhancer of activated B cells	NK-kB
Oral glucose tolerance test	OGTT
Oncostatin	OSM
Pathogen associated molecular patterns	PAMPs
Pattern recognition receptor	PRR
Penicillin	PEN
Peripheral blood mononuclear cells	PBMCs
Peroxisome proliferator-activated receptor-γ	PPARγ
Phorbol myristate acetate	PMA
Phosphate buffered saline	PBS
Phytohaemagglutin	PHA
Polymerase Chain Reaction	PCR
Poorly controlled type 2 diabetes	pT2D
Positron emission tomography–computed tomography	PET-CT
Pre type 2 diabetes	PreT2D
Promyelocytic leukemia zinc-finger	PLZF
Prothionamide	Pto
Purified protein derivatives	PPD
Pyrazinamide	PZA
Quality control	QC
Quantiferon	QFT
RAR-related orphan receptor γt	RORγt

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Region of difference-1	RD-1
Rifampicin	Rif
Rifampicin resistance TB	RR-TB
Room temperature	RT
Selective sodium-glucose transporter-2	SGLT-2
Serum amyloid protein –A	SAP-A
Sodium heparinized	NaHep
Streptozotocin	STZ
Sub Saharan Africa	SSA
Tetramethylbenzidine	TMB
T cell receptor	TCR
Thawing media	TM
Thiazolidinediones	TZDs
Thyroid-stimulating hormone	TSH
T helper	Th
Toll like receptor	TLR
Transforming growth factor	TGF
Transient hyperglycaemia	THG
Tuberculin skin test	TST
Tuberculosis	TB
Tuberculosis and diabetes comorbidity	TB-T2D
Tumour necrosis factor	TNF
Type 2 diabetes	T2D
Type 1 diabetes	T1D
Vasopressin	VP
World Health Organization	WHO
5 Hydroxytryptamine-1	5 HT-1
α-galactosylceramide	α-Gal-Cer

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CHAPTER 1

Literature review and introduction

1. Diabetes

1.1 Diabetes prevalence

Diabetes mellitus (DM) is a chronic disease that occurs either because there are defects in beta cell function in the pancreatic islets, which then cannot produce adequate levels of insulin, like in type 1 diabetes (T1D), or the produced insulin cannot be utilized by insulin responsive tissues such as muscles, liver and adipocytes, like in type 2 diabetes (T2D). T2D manifests due to insulin resistance and/or pancreatic β-cell failure (decreased/no insulin secretion). In both cases, hyperglycemia occur, meaning individuals with T2D may still be secreting insulin, but do not respond to the insulin due to the increased insulin resistance. Chronic hyperglycaemia subsequently decreases beta cell function and insulin secretion as a result of the glucotoxicity, lipotoxicity, and enhanced oxidative stress (Guillausseau et al. 2008; Upadhyay et al. 2017). T1D is the least prevalent form of diabetes and was, for many years, classified as the inherited form of the disease until it became evident that genetic factors and auto-immune reactions play a role in the development of T1D (Florez et al. 2003).

In 2017, it was estimated that about 425 million people of the age group 20-79 years have T2D and this number is expected to reach 629 million by 2045 (Cho et al. 2018). Approximately five million people died due to T2D, globally in 2017 (Cho et al. 2018). Asia contributes to about 60% of the global T2D prevalence with India having about 62 million people living with T2D and more than 77 million with pre-diabetes (Kaveeshwar and Cornwall, 2014). Of the latter, 70% will continue to develop T2D (Tabák et al. 2012). Pre-diabetes refers to impaired glucose tolerance, impaired fasting glucose (IFG) or intermediate hyperglycaemia (Echouffo-Tcheugui and Dagogo-Jack, 2012). It is associated with micro-and macro-vascular complications, as well as an increased risk to progress to T2D (Brannick et al. 2016). It is therefore important to closely monitor patients with pre-diabetes in an attempt to prevent them from developing T2D. In Sub-Saharan Africa (SSA), there is an estimated 15.5 million people with T2D between the ages of 20 and 79, and 1.8 million of those reside in South Africa (International Diabetes Federation, 2017). An estimated 69.2% of people living with T2D in SSA are undiagnosed and therefore at higher risk of developing harmful and costly complications (International Diabetes Federation,2017). In South Africa, the prevalence of self-reported T2D is 9.2% and increases with age: from 7.1% amongst 50–59-year olds to 10.6% amongst 60–69-year olds and 12.4% amongst those 70 years and older (Werfalli et al. 2018). In the Western Cape Province of South Africa, the highest prevalence of T2D has been reported in the Coloured population (Erasmus et al. 2012), who also has an undiagnosed T2D prevalence of 18.1% (Erasmus et al. 2012). This suggests that there might be even more

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people with T2D because there are patients with T2D who remain undiagnosed and who are unaware of the damage caused by the disease (International Diabetes Federation, 2015). As a result, controlling this disease will be more difficult. As urbanisation increases and populations age, T2D will pose an ever-growing threat in these communities (International Diabetes Federation, 2017).

1.2 Diagnosis of diabetes

Measurements of plasma glucose and glycated haemoglobin A1c (HbA1c) are used as diagnostic tools for T2D (Florkowski, 2013). The World Health Organization (WHO) together with the National Diabetes Data Group in the US recommend the use of the oral glucose tolerance test (OGTT) measuring both fasting and two hours plasma glucose concentrations (National Diabetes Data Group, 1979). According to the American Diabetes Association (ADA) a fasting blood glucose level (FBG) of ≥ 7.0 mmol/L or a blood glucose level of ≥ 11 mmol/L after ingesting 75 g of glucose indicates that a person has DM (ADA, 2011). Impaired glucose tolerance (IGT) is characterised by a blood glucose level between 7.8 and 11 mmol/L, after ingesting glucose, and impaired fasting glucose (IFG) as a FBG between 6.1 and 6.9 mmol/L (ADA, 2011). The WHO recommends a FBG cut off of 7 mmol/L for diagnosis of DM (ADA, 2011). Both the ADA and WHO recommended HbA1c, with a cut off of ≥ 6.5%, as an additional test to diagnose DM (International expert committee, 2009; WHO, 2011), however, OGTT remains the gold standard.. Unlike real time glucose measurements, HbA1c gives an indication of average blood glucose concentrations over two to three months period (Reynolds et al. 2006). The advantage of HbA1c measurements is that individuals are not required to be fasted, it requires less time to perform compared to an OGTT and the pre-analytical stability of HbA1c is high. Despite the advantage of HbA1c, the levels are known to vary in racial groups, with Black Africans having higher HbA1c compared to caucasians (Herman et al. 2007; Kirk et al. 2013). In African Americans, changing from using glucose to HbA1c, resulted in about 650 000 missed T2D cases (Paterson, 2017). Furthermore, the glucose-6-phosphate dehydrogenase (G6PD) variant rs1050828 is common in people of African and African American decent and impacts HbA1c outcome, which if not recognised, can result in undiagnosed T2D when using HbA1c (Paterson, 2017). People with hemizygous T, heterozygous and homozygous T allele were found to have low HbA1c compared to those without T and also had lower red blood cell counts, hemotocrit and haemoglobin. It was suggested that the effect of rs1050828 variant on HbA1c is likely through reducing red blood cell lifespan (Paterson, 2017). As G6PD deficiency can be clinically ‘silent’ until illness strikes,some researchers suggest looking into the possible benefits of screening the G6PD genotype in parallel to testing HbA1c to diagnose T2D, especially in populations with African ancestry or population groups where the G6PD deficiency is common to avoid missed or

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delayed diagnosis of the disease (Wheeler et al. 2017). Although the OGTT is the gold standard for T2D diagnosis, it is more challenging to perform as it requires overnight fasting (Florkowski, 2013). Other pre-test preparations are recommended such as ensuring that an appropriate diet was followed for three days prior to the test (Florkowski, 2013). The test itself is time-consuming, taking at least two hours. In addition, when measuring plasma glucose concentrations, blood should be processed within several minutes after collection, because red blood cells continue to consume glucose which could lead to false negative results (Bruns and Knowler, 2009). Missed T2D cases could be due to the lack of standardized testing for T2D (Matsha et al. 2016) and more studies should focus on diagnostic tools to improve certainty of the estimates of T2D prevalence. Earlier detection of T2D also has a benefit on the management of disease outcomes such as glycaemic control, which in turn will reduce the risk of developing T2D complications.

1.3 Diabetes treatment

Traditionally, diet modifications have been the basis for DM management. There are several classes of medications for the treatment of DM, which are used as an adjunct to diet and exercise. These include biguanides, sulfonylureas, meglitinide derivatives, alpha-glucosidase inhibitors, thiazolidinediones (TZDs), glucagon like peptide–1 (GLP-1) agonists, dipeptidyl peptidase IV (DPP-4) inhibitors, selective sodium-glucose transporter-2 (SGLT-2) inhibitors, and insulin (National diabetes service scheme, 2016). It is important that T2D medications be reviewed by patients' general practitioner every year as part of the T2D annual cycle of care (National diabetes service scheme, 2016).

Biguanides are the first line treatment for T2D, an example is metformin (Inzucchi et al. 2015). Metformin mediates its effects through two mechanisms to improve blood glucose concentration, it inhibits gluconeogenesis and enhances peripheral insulin sensitivity (Goodarzi and Bryer-ash, 2005). A dose of 500 mg metformin is initially prescribed to patients, once or twice a day with breakfast or/and dinner and may be increased after a week to 850 to 1000 mg, twice a day, depending on how the patient responds to the treatment and whether the patient experiences any side effects (Upapdyay et al. 2017). Metformin can be used alone or in combination with sulfonylureas, TZDs or insulin (Khardori, 2018). On its own, it reduces HbA1c by an average of 1.5% (Knowles et al. 2002) and reduces the progression of pre-diabetes to T2D by 31%. Thus, it is thought to be a good prophylaxis for T2D in individuals with pre-diabetes (Inzucchi et al. 2015). Metformin also reduces body weight and in combination with insulin therapy, reduces complications associated with DM such as macrovascular disease, heart failure, stroke and other peripheral artery diseases (Kooy et al. 2009). Sulfonylureas are the second most prescribed drugs for T2D. They stimulate the pancreatic beta cells to secrete insulin (Mendez et al. 2014). These drugs are associated with

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a reduction of HbA1c by 1-2% and blood glucose levels of about 20% (Bolen et al. 2006). They are used both as monotherapy and in combination with insulin and other oral drugs for treating hyperglycaemia. An example is Glimepiride which is the only sulfonylurea approved by the food and drug administration (FDA) for combination therapy. Other examples include Glyburide and Glipizide. Meglitinide functions similarly to sulfonylurea by inducing the production of insulin by the pancreatic beta cells and examples include Repaglinide and Nateglinide (Melander, 2004). These drugs are FDA approved for monotherapy use and in combination with Metformin or TZDs (Khardori, 2018). TZDs include Pioglitazone and Rosiglitazone. These drugs improves the target cell responses to insulin by stimulating glucose uptake through the nuclear receptor called peroxisome proliferator-activated receptor-γ (PPARγ) in skeletal muscles and adipose tissue and as a result, lower glucose plasma levels (Ahmadian et al. 2013). TZDs can be used as monotherapy and in combination with sulfonylureas and/or metformin and insulin (Khardori, 2018). Alpha-Glucosidase inhibitors promote the body to break down starchy foods and sugar, examples include Acarbose and Miglitol (Mendez et al. 2014). These drugs lower the blood glucose levels. They can be used as monotherapy or in combination with sulfonylurea (Khardori, 2018). Incretin based therapies consists of GLP-1 receptor agonists and DPP-4 inhibitors (Inzucchi et al. 2012). GLP-1 receptor agonists are injectable drugs mimicking the endogenous GLP-1 which stimulates glucose-dependent insulin release to normalize hyperglycaemia (Meier, 2012). Exenatide, Albiglutide and Liraglutide are some of the examples of GLP-1 analogues and function by improving glycaemic control. Exenatide is administered twice daily while Albiglutide and Liraglutide are administered once daily (Khardori, 2018). DPP-4 is an endogenous metabolic enzyme that is involved in the regulation of glucose homeostasis if inhibited (Meier 2012). DPP-4 assists the body to continue releasing insulin by increasing the plasma levels of GLP-1 through the inhibition of its proteolytic degradation (Meier, 2012). DPP-4 include drugs such as Sitagliptin, Saxagliptin, Linagliptin and Alogliptin. DPP-4 inhibitors can be used in combination with metformin, TZDs and sulfonylurea (Khardori, 2018). SGLT-2 inhibitors are agents that lower the glucose concentrations in the kidneys by increasing urinary glucose release (Ferrannini and Solini, 2012). Examples include Canagliflozin, Dapagliflozin and Empagliflozin (Khardori, 2018).

In T2D, the use of insulin is only prescribed when beta cells are exhausted (Li et al. 2015). Insulin treatment is divided into three categories according to their mechanism of action, which are basal (long-acting), prandial (rapid-acting) or pre-mixed (mix of long- and rapid-acting). Basal insulin treatment uses a human preparation called protamine Hagedorn (NPH) as well as other insulin analogues. The analogues include detemir and glargine. The insulin analogues have a prolonged action and result in lower hypoglycaemia rates compared to NPH.

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Their HbA1c reducing abilities are however comparable. The analogues regulate early postprandial hyperglycaemia and late postprandial hypoglycaemia (Hirsch, 2005). The use of pre-mixed insulin, which is a combination of basal and prandial insulin, is convenient as it reduces the number of daily insulin injections and simplify insulin treatment. Studies comparing the three insulin types show that the HbA1c lowering capabilities are comparable while basal insulin reduces the risk of hypoglycaemia and weight gain (Holman et al. 2009).

2. Tuberculosis

2.1. Tuberculosis prevalence

Tuberculosis (TB) is a disease caused by *Mycobacterium tuberculosis* (Mtb). The bacteria primarily affect the lungs of the infected individual. Despite being a curable disease, TB remains one of the top 10 causes of death globally (WHO, 2018). In 2017, there were 10 million incident cases of TB in the world and 1.3 million people died because of TB (Figure 1.1) (WHO, 2018). About 82% of TB deaths occurred in the African Region and the South-East Asia Region in 2017 (WHO, 2018). South Africa has the third highest incidence of TB following China and India and in 2017 the WHO estimated the incident of TB to be 500 000 in South Africa (Figure 1.1) (WHO, 2018). It is estimated that 80% of the South African population is latently infected with Mtb (Kanabus, 2017).

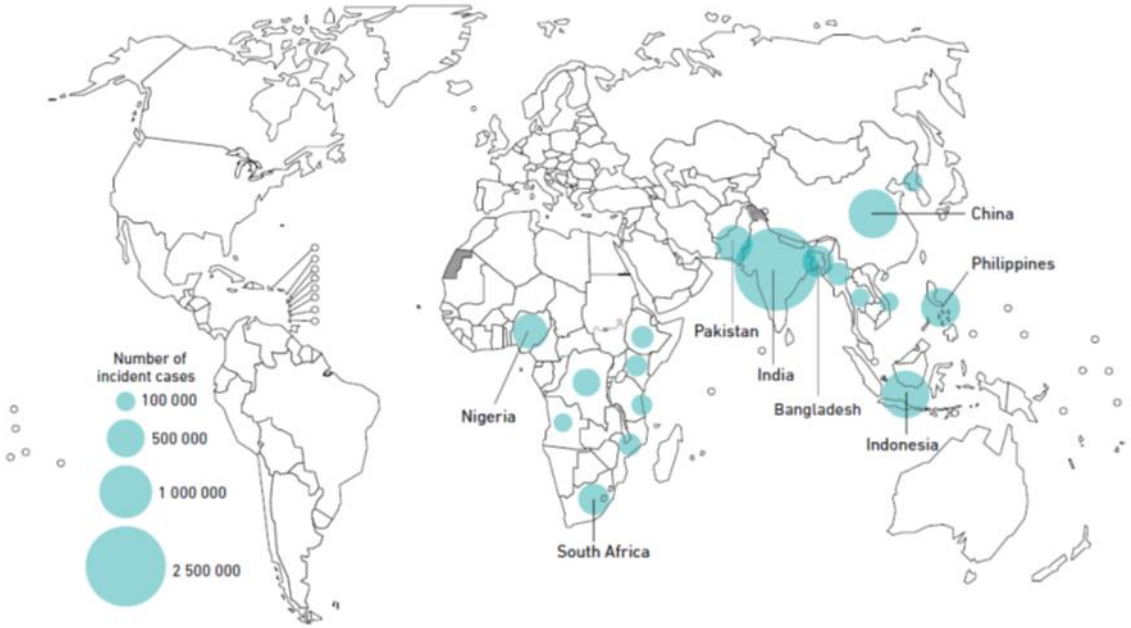

Figure 1.1: TB incidence of the highest TB endemic areas in the world reported in 2017 (WHO, 2018).

Approximately 25% of people worldwide are infected with Mtb (Houben and Dodd, 2016). In most individuals Mtb remains in a latent, asymptomatic state called latent TB (LTBI) and approximately 5-10% progress to the active, symptomatic form of TB within the first two years after infection (Comstock et al. 1974; Lillebaek et al. 2002). Latently infected individuals are at

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increased risk of developing active TB when their immune systems becomes compromised due to infection with human immunodeficiency virus (HIV) (Wood et al. 2011) or have T2D (Jeon and Murray, 2008).

In addition, there are about 157 505 people in South Africa who do not know that they have TB (WHO, 2018) and who unknowingly continue to spread the disease. Approximately 54 million lives were saved through TB diagnosis and treatment between 2000 and 2017 (WHO, 2018), which shows the importance of identifying and treating patients as a means to control the TB epidemic. Furthermore, to prevent and control TB disease, *Mycobacterium bovis* Bacillus Calmette-Guerin (BCG) is widely used as vaccine to prevent TB meningitis associated deaths in infants and young children (Trunz et al. 2006), however, it does not prevent the development of pulmonary TB in adults (Pai et al. 2016). Emphasis has therefore been placed on the development of more efficacious vaccines to prevent TB disease in adults.

2.2 Diagnostic tools for TB infection and disease

The successful management of TB depends on the ability to detect patients with the disease and initiating TB treatment. Currently, there are no diagnostic tools that can accurately differentiate between LTBI and TB disease (Tebruegge et al. 2010). Methods widely used to diagnose TB include microbiological, molecular as well as immunological tests and X-ray.

2.2.1 Immunological tests determine whether a patient's T cells have been sensitized to Mtb specific antigens (Andersen et al. 2000). The Tuberculin skin test (TST) using purified protein derivatives (PPD) is used to test for Mtb infection. The procedure involves injecting tuberculin PPD under the skin of the inner forearm. If the individual was exposed or infected with Mtb and has a memory T cell response, an induration ≥ 5 mm in children and HIV positive individuals and ≥ 10 mm in other high risk groups will form after 48–72 hours (CDC, 2016). It is however less specific as it can result in false positive readouts due to repeated TST testing, antigenic cross reactivity with BCG after vaccination or infection with non-tuberculous mycobacterium (NTM) (Tebruegge et al. 2010).

Interferon-gamma (IFN-y) release assays (IGRA) such as the Quantiferon (QFT) TB Gold in-tube, QFT TB Gold Plus and T-SPOT TB test are also used for the diagnosis of Mtb infection. These assays measure cell mediated immune responses in individuals suspected of being infected with Mtb. The QFT TB Gold in-tube test consists of three tubes coated with the following; a nil tube, containing no additives and serves as negative control to adjust for background IFN-y production, a TB-antigen tube, coated with an antigen cocktail containing region of difference-1 (RD-1) encoded Mtb antigens (early secretory antigen-6 (ESAT-6), culture filtrate protein-10 (CFP-10) and TB 7.7) and a mitogen tube, coated with phytohaemagglutin-P (PHA) that serves as a positive control. Whole blood is incubated

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overnight in the three tubes during which $CD4^+$ T cells are stimulated to release IFN-y (Pai et al. 2008), which is measured by ELISA (Yi et al. 2016). The QFT TB Gold Plus assay utilizes two antigen tubes. The TB-1 antigen tube contains larger CFP-10 and ESAT-6 peptides that stimulates $CD4^+$ T cells and the TB-2 antigen tube contains shorter peptides to stimulate $CD8^+$ T cells, resulting in an increase in the sensitivity of the test (Yi et al. 2016; Losi et al. 2016). $CD8^+$ sensitization is important because the IFN-γ is not only produced by $CD4^+$ cells, also $CD8^+$ cells (Petruccioli et al. 2016). $CD8^+$ T cell activation is useful in immunocompromised individuals where there are poor $CD4^+$ responses (Petruccioli et al. 2016).

Similarly to the QFT, the T-SPOT TB assay measures IFN-y produced by activated T cells after Mtb antigen stimulation and by doing so indicates whether a person has developed a memory T cell response against Mtb (Hill et al. 2005). PBMCs are isolated and incubated with ESAT-6 and CFP-10 in plates coated with anti-IFN-y antibodies (Wang et al. 2007). The IFN-y secreted by sensitized T cells is captured by specific antibodies on the plate and presented as spots (Oxford Immunote Ltd, Edinburgh, UK; Zhu et al. 2014). Depending on whether a patient presents with symptoms associated with TB or show lesions suggesting TB on chest X-ray or computerised tomography (CT) scan result, they will be diagnosed as either being LTBI or having TB when their T-SPOT result is positive (CDC, 2000).

2.2.2 Microbiological tests include staining for acid-fast bacilli in sputum smear as well as culturing of sputum samples. These techniques are not specific to Mtb and are time consuming (Murray and Thompson, 1980). Direct sputum smear microscopy utilizes the Ziehl Nielsen acid-fast staining to visualize Mycobacterial species including Mtb, *M. ulcerans, M. leprae* and NTMs using a microscope. A minimum of 5000 bacilli/mL are required for the test to be positive (Murray and Thompson, 1980). Therefore, a negative smear does not necessarily rule out TB and a positive smear does not confirm Mtb infection but rather the presence of Mycobacterial species.

The mycobacteria growth indicator tube (MGIT) automated mycobacterial detection system (BD BACTEC MGIT 960 system, BD) is the gold standard for TB diagnosis and requires at least 10 bacilli/mL for a positive diagnosis (Siddiqi and Rusch-Gerdes, 2006). The decontaminated sputum sample is inoculated in the tube and then incubated at 36°C and monitored continuously by a BACTEC 960 instrument for 42 days before the test can be deemed negative. The MGIT system allows the detection of mycobacterial growth using a fluorescent indicator embedded in silicone at the bottom of the tube. The fluorescent indicator is quenched by oxygen present in the broth. Once the growing mycobacteria replicate and utilise the available oxygen, the unmasked fluorescence is detected by the system and flagged as positive (O'Brien, 2016).

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2.2.3 Molecular test

The GeneXpert MTB/RIF assay (GeneXpert MTB/RIF; Cepheid, Sunnyvale, CA) is a molecular test based on the Polymerase Chain Reaction (PCR) principle. The GeneXpert MTB/RIF assay was developed in 2009 to simultaneously diagnose Mtb and rifampicin (RIF) drug resistance within two hours (Blakemore et al. 2010). This test is recommended for use as an initial diagnostic tool when MDR-TB is suspected and during HIV-TB co-infection (WHO, 2010), but is not yet a replacement for culture. In March 2017, the GeneXpert Ultra (GeneXpert MTB/RIF Ultra, Cepheid) was launched which has an increased sensitivity that is comparable to that of the MGIT (WHO, 2018). The WHO recommendations for the use of Xpert MTB/RIF also apply to the use of Ultra as the initial diagnostic test for all adults and children with signs and symptoms of TB (WHO, 2018).

2.3. Treatment of TB

LTBI treatment is not common in South Africa, because individuals with LTBI are likely to become re-infected with Mtb in such a high TB burden setting, and therefore prophylactic treatment is not only not effective (Churchyard et al. 2012), but may also fuel drug resistance. Prophylactic treatment is only given to children below the age of five years who are close contacts (CCs) of TB patients (WHO, 2018), individuals with LTBI who are HIV positive, women who are pregnant and individuals with other immunosuppressive conditions (Leutkemeyer, 2013). In low TB incident countries, on the other hand, preventative treatment is given to individuals with LTBI to prevent the development of TB (Lönnroth et al. 2015). The preventative therapy options includes a weekly dose of Rifapentine and INH for three to four months, a daily dose of RIF plus INH for three months or a daily dose of RIF for three to four months (WHO, 2018).

2.3.1 The standard TB treatment reduces the number of actively growing bacteria, prevents transmission of the bacilli and prevents death (Nahid et al. 2016). The standard first line treatment regimen for new and previously treated TB patients includes Ethambutol (EMB), INH, RIF and Pyrazinamide (PZA). Standard TB treatment consists of two phases; an initial two months intensive phase where EMB, INH, RIF and PZA are given followed by a continuation phase of four months where INH and RIF are given (WHO, 2018). Directly observed treatment short course (DOTS) is a strategy used, where patients are observed swallowing their medication to ensure completion of therapy (Nahid et al. 2016).

2.3.2 The treatment regimen for drug resistant TB, including RIF resistant TB (RR-TB), multi-drug resistant TB (MDR-TB) and extensively drug resistant TB (XDR-TB) is different from the standard TB treatment and includes second-line drugs. MDR-TB occurs when Mtb is resistant to RIF and INH and both RR-TB and MDR-TB requires treatment with second-line drugs (WHO, 2018). XDR-TB occurs when Mtb acquires resistance to INH, RIF, at least one

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fluoroquinolones (Levofloxacin and Moxifloxacin (Mfx)) and a second-line injectable drug (Amikacin, Capreomycin or Kanamycin) (WHO, 2018). In 2018, the WHO convened a Guideline Development Group who is revising the guidelines of the drug resistant TB regimen including the use of the shorter nine months MDR-TB regimen (WHO, 2018). The new guidelines will be released late in 2018. Medicines to select as first priority include the later generation of fluoroquinolones (levofloxacin or moxifloxacin), bedaquiline and linezolid. Other agents are included based on their relative potency. The use of injectable drugs is not required anymore and longer oral-only regimens are still available for many patients. The older injectable drugs, kanamycin and capreomycin, are no longer recommended for use by the WHO due to their association with severe side effects such as nephrotoxicity and ototoxicity.

3. Latent Mtb infection and T2D

Participants who are latently infected with Mtb and who have T2D are at a three times increased risk of developing TB compared to a healthy individual with LTBI (Jeon and Murray, 2008). Therefore, screening TB contacts for T2D is effective for identifying new T2D cases at high risk of developing TB (Restrepo et al. 2018). We enrolled 95 contacts after screening 247 individuals for T2D in South Africa and demonstrated that 17.4 % of TB contacts in our South African cohort have T2D and almost half of these individuals (43.9 %) are not aware that they have this disease (Restrepo et al. 2018). The factors associated with T2D such as hyperglycaemia and dyslipidaemia may play a role in the altered immune function associated with T2D, increasing the risk of TB development in individuals with LTBI and T2D (Restrepo et al. 2018). The immune profile of individuals with LTBI and T2D compared to LTBI without T2D have been characterized by reduced T helper 1 (Th1), Th17 and Th2 associated cytokines (Kumar et al. 2014; Figure 1.2). Cytokines associated with Th1 (interleukin-2 (IL-2), IFN-γ, Tumour necrosis factor- α (TNF-α) and Th17 (IL-17 A, IL-17F, IL-22) cells as well as the IL-1 family (IL-1α, IL-1β, IL-18) are important in resistance to TB (Cooper and Khader, 2008; O'Garra et al. 2013). In plasma of individuals with LTBI and T2D (LTBI-T2D), IL-2, IFN-y and TNF-α were significantly lower compared to LTBI (without T2D) (Kumar et al. 2014). IL-17F was significanlty lower while IL-22 was significantly higher in LTBI-T2D compared to LTBI. IL-1 family cytokines such as IL-1β and IL-18 were lower in LTBI-T2D compared to LTBI and IL-1α was not different between the two groups (Kumar et al. 2014). The pro-inflammatory cytokines, IL-6, IL-12 and granulocyte macrophage-colony stimulating factor (GM-CSF) measured in plasma were the same in both groups (Kumar et al. 2014). Th2 (IL-4, IL-5, IL-13) associated and regulatory (IL-10, transforming growth factor-β (TGF-β)) cytokines, which play a role in the progression to TB disease (O'Garra et al. 2013), were not significantly different in plasma between LTBI-T2D and LTBI (Kumar et al. 2014). Only IL-10 was significantly lower

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in plasma of LTBI-T2D patients compared to individuals with LTBI. The concentration of IFN-γ, TNF-α, IL-17A and IL-10 measured in whole blood stimulated with Mtb antigens (CFP-10, ESAT-6 and TB7.7), using the QFT-intube assay, are significantly lower in LTBI-T2D patients compared to individuals with LTBI (Kumar et al. 2014).

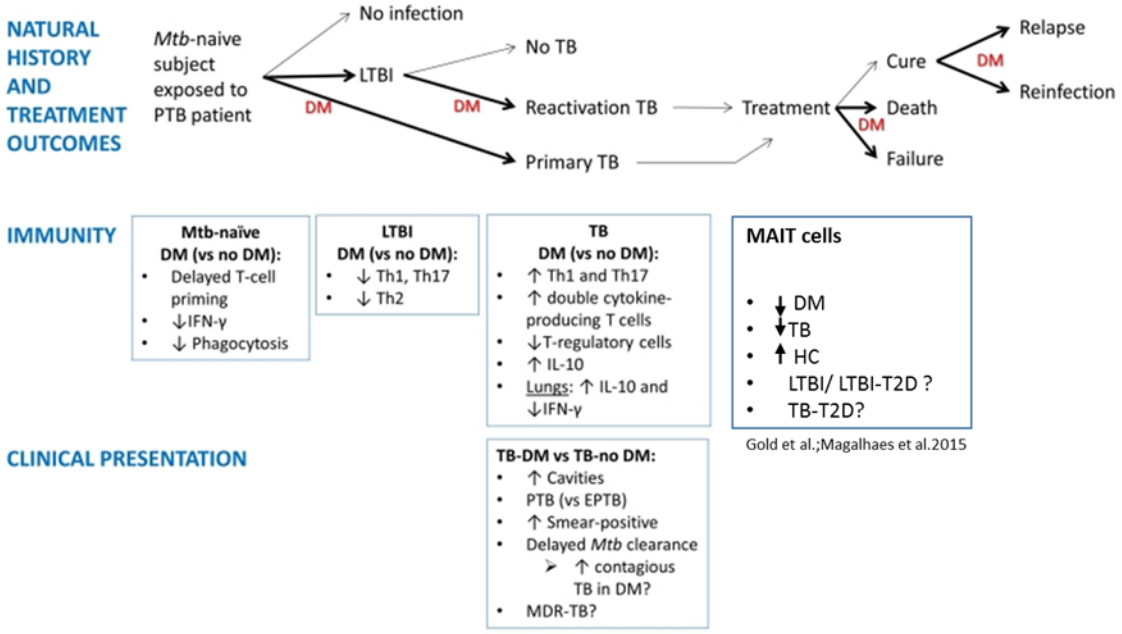

Figure 1.2 The influence of T2D on the onset of TB, dysregulated immune response and clinical presentation of the TB during TB-T2D comorbidity (adapted from (Restrepo, 2016)).

4. TB and T2D

Globally, 790 000 TB cases were attributable to T2D and 880 000 people who had HIV also had TB (WHO, 2018). HIV infection is accountable for 8.7% and T2D for 7.5% of TB cases in the world in 2017 (WHO, 2018). However, since there are about 36 million HIV infected people and 460 million people with T2D (WHO, 2018), it is said that there will be more TB-T2D cases than TB-HIV (Lonnroth et al. 2014). About 75% of all people with T2D live in low- and middle-income countries, where TB also flourishes (Kapur et al. 2016). About 25% of TB cases in India has T2D and 50% stress induced hyperglycaemia (Viswanathan et al. 2012). Among the TB patients with T2D, approximately 60% were previously diagnosed with T2D, which may have a negative effect on the immune response and could enhance TB morbidity (Viswanathan et al. 2012). In South India in 2011, T2D was a greater risk factor for TB (30.9%) than HIV (10.6%) (Gupta et al. 2011). A Mexican study showed that 39% of patients with T2D had TB, which is roughly five times more than TB cases attributable to HIV (Restrepo et al. 2011). In SSA, where the TB incidence is high, little is known about how T2D affects the TB burden (Skowroński et al. 2014), and there is an urgent need for such studies. Stopping the

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rise of T2D globally would avoid 6 million new incident cases and 1.1 million TB deaths during a 20 year period (Pan et al. 2015). Should interventions reduce the T2D incidence by 35%, 7.8 million TB cases and 1.5 million TB deaths could be averted by 2035 (Pan et al. 2015). An estimated increase in T2D in TB endemic areas contributes to the challenge to control TB disease (International Diabetes Federation, 2015; Pereira et al. 2016). Patients with poorly controlled T2D, in particular, are at increased risk of developing active TB (Baker et al 2012) and are at increased risk of poor TB treatment outcome and death (Jeon and Murray, 2008; Dolley et al. 2009; Restrepo et al. 2011) (Figure 1.2).

Although studies have generally identified T2D as a risk factor for TB disease development rather than TB disease as a risk factor for the onset of T2D (Stevenson et al. 2007; Harries et al. 2010), we cannot rule out this possibility, as a contributing factor to the increase in TB-T2D cases (Critchley et al. 2017), as TB (in absence of T2D) is well known to be associated with transient stress induced hyperglycaemia (Magee et al. 2018). T2D is not only associated with TB disease progression from LTBI, but is also linked to more severe clinical presentation of TB disease. Patients with TB-T2D are more likely to present with pulmonary and not extra-pulmonary TB, have more cavities, increased TB severity scores, they are contagious for longer due to delayed culture conversion rates and at increased risk of MDR-TB (Fisher-Hoch et al. 2008; Gil-Santana et al. 2016; Liu et al. 2017) (Figure 1.2; Restrepo, 2016). The latter is somewhat controversial as some studies do not show an association between T2D and MDR-TB (Magee et al. 2013; Chiang et al. 2015). TB-T2D patients compared to TB with no T2D are characterized by alterations in Th1 and Th17 cytokines (Kumar et al. 2013). Th1 (IL-2, IFN-y, TNF-α), Th17 (IL-17A, IL-22) and regulatory (IL-10 and TGF-β) cytokines were previously compared between TB-T2D and TB patients (Kumar et al. 2013). IL-22 was significantly lower in TB-T2D whereas IL-2, IFN-y, IL-17, IL-10 and IL-5 were significantly higher in TB-T2D in comparison to TB patients (Kumar et al. 2013a; Kumar et al. 2013b). There was no difference in the concentration of TNF-α and TGF-β between the two groups. In addition, IL-1 family members (IL-1β, IL-18) and other pro-inflammatory (IL-6) cytokines were higher and type 1 interferon (IFN-β) lower in the plasma of TB-T2D patients compared to TB patients (Kumar et al. 2013). IL-12, GM-CSF, IL-1α, IFN-α, IL-8, Chemokine (C-C) motif ligand 11 (CCL11), CCL2, CCL4, granulocyte colony–stimulating factor (G-CSF), Chemokine (C-X-C) motif ligand CXCL10 and platelet derived growth factor (PDGF) were not significantly different between the two groups (Kumar et al. 2013).

In unstimulated serum (obtained from the QFT in-tube assay), IFN-y, TNF-α, IL-17 and IL-1 were significantly elevated in TB-T2D patients compared to TB patients. Similarly, the same was seen after measuring the cytokines in Mtb-antigen stimulated serum. IL-10 was also significantly higher in TB-T2D compared to TB, but IL-1β was not different between the groups

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in this sample type (Kumar et al. 2013). When using broncho alveolar lavage (BAL), which is a representative sample of the site of disease, IFN-γ is higher and IL-10 lower in TB-T2D compared to TB (Sun et al. 2012).

5. Innate immunity

5.1 Innate immunity in TB

The innate immune response during Mtb infection is important and some individuals are able to clear the infection without developing an adaptive immune response to Mtb, also referred to as early clearance (EC) (Verrall et al. 2013). After EC, the TST and/or IGRA tests remain negative because the T cells are not primed (Figure 1.3). If EC is not achieved, $CD4^+$ T cells secreting IFN-γ are subsequently recruited and the adaptive response initiated (Verrall et al. 2013). Once T cells are primed and effector memory T cells against Mtb antigens are in circulation, the TST or IGRA becomes positive (Figure 1.3). The major host innate immune cells that are important during Mtb exposure are macrophages, dendritic cells (DCs), neutrophils and the natural killer (NK) cells (Sia et al. 2015). Mtb interacts with the receptors expressed on the surface of these innate cells called pattern recognition receptors (PRRs) such as toll-like receptors (TLRs), Nod-like receptors (NLRs) and C-type lectin receptors (CLRs) (Killick et al. 2013).

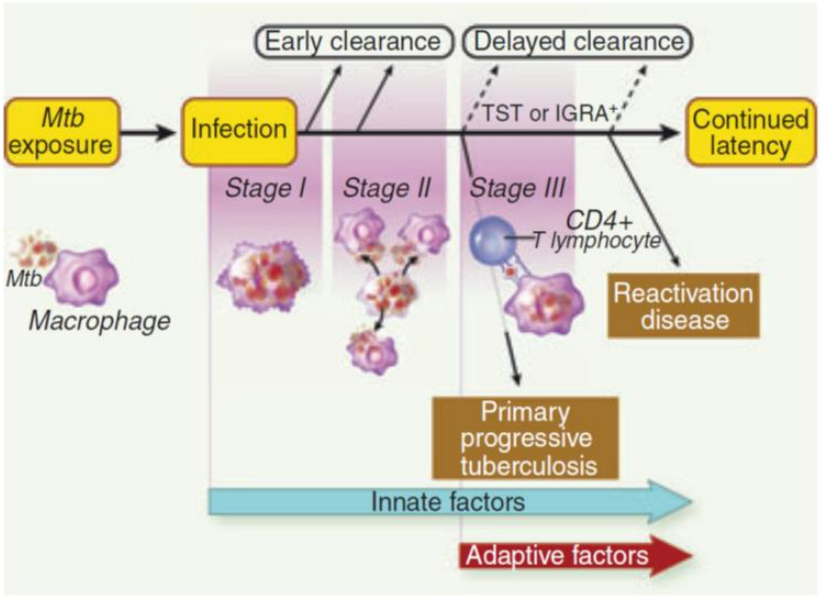

Figure 1.3: Possible outcomes after Mtb exposure: 1) Stage I and stage II showing early clearance which involves innate immune response, and no apparent adaptive immune response, resulting in IFN-γ release assay (IGRA) and tuberculin sensitivity test (TST) negativity, 2) stage III involving adaptive immune response and delayed clearance or latent infection shown by IGRA/TST positivity (adapted from Verrall et al. 2014).

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Macrophages are known to be the primary defence cell type during the initial encounter with Mtb. Mtb pathogen associated molecular patterns (PAMPs) such as glycolipids, lipoproteins and carbohydrates are recognized by PRRs on macrophages and induce a network of signalling pathways that will favour antimicrobial effector functions within macrophages (Sia et al. 2015). The mechanisms used by macrophages to eliminate Mtb involve inflammasome activation, production of oxygen and nitrogen components, phagosome acidification, autophagy of intracellular Mtb and apoptosis (Cadena et al. 2016). IFN-y plays an important role in host response against Mtb infection and activates macrophages to kill and restrict Mtb growth. In addition, IFN-γ induces the production of cytokines such as IL-12 and TNF-α by macrophages and other myeloid cells while inhibiting IL-10 production (Flynn et al. 1993; MacMicking et al. 1997; Liu et al. 2003; Mcnab et al. 2014). Type 1 interferons; IFN-α and IFN-β are associated with unfavourable outcomes during Mtb infection (McNab et al. 2014) through inhibition of macrophages responsiveness to IFN-y and subsequent activation (Mcnab et al. 2014). Studies have shown decreased bacterial load and increased survival of mice when IFN-α and IFN-β signalling are inhibited (Manca et al. 2005; Ordway et al. 2007; Stanley et al. 2007). Mtb infected IFN-αR1 knock out mice had a significantly lower bacterial burden compared to wild type mice (Mcnab et al. 2014). When knock out mice were treated with both IFN-β and IFN-y, no change in bacterial load was observed, suggesting that type I IFNs inhibit macrophage restriction of Mtb growth and killing in response to IFN-y (Mcnab et al. 2014). Type I IFNs may therefore act to interrupt Th1 immune response, by making macrophages unresponsive to IFN-y and subsequently suppressing production of pro-inflammatory cytokines. Additionally, type I IFNs may act directly to prevent IFN-y mediated killing of Mtb by macrophages, allowing Mtb to replicate within these cells (Mcnab et al. 2014). The role of type 1 IFNs is not universally observed, type 1 IFNs were shown to reduce the number of macrophages in the lung, reducing Mtb target cells in the lung, while addition of IFN-γ enhances their ability to restrict bacterial Mtb growth (Desvignes et al. 2012). It is speculated that the contradicting results are due to the different Mtb strain used and differences in study designs (Mcnab et al. 2014).

DCs are key players in innate immunity during Mtb infection (Bhatt and Salgame, 2007) and are important for the transitioning from innate to adaptive immune response (Prendergast and Kirman, 2013). They are antigen presenting cells (APCs) and secrete cytokines that activate naive lymphocytes (Mellman et al. 2001; Sia et al. 2015). DCs secrete IL-12 (Giacomini et al. 2001), which subsequently activate NK cells and T cells to produce IFN-y. DC-specific ICAM-grabbing nonintegrin (DC-SIGN) is the predominant receptor on human DCs that recognises Mtb (Tailleux et al. 2003). The mycobacterial macrophage receptors such as complement receptor (CR3) and mannose receptor (MR) play a minor role in the recognition of

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mycobacterial ligand lipoglycan lipoarabinomannan (LAM) by DCs. This may be due to the greater abundance of DC-SIGN on DCs relative to CR3 and MR (Tailleux et al. 2003).

Tailleux et al. investigated DC-SIGN trafficking in DCs infected with GFP-expressing Mtb *H37Rv* using confocal microscopy (Tailleux et al. 2003). During early phagocytosis (first hour), most bacilli were detected on the cellular membrane of DCs or co-localized with DC-SIGN in nascent phagosomes. DC-SIGN staining was not detected on phagosomes that had detached from the plasma membrane, indicating that it was excluded from the vacuoles immediately after phagocytosis (Tailleux et al. 2003). It is thus thought that DC-SIGN is rapidly expelled from the phagosome and recycled to the cell membrane (Tailleux et al. 2003). The intracellular trafficking pattern of DC-SIGN in Mtb infected DCs could also indicate that DC-SIGN transports the mycobacterial glycolipids from the bacterial vacuole to various subcellular compartments, where glycolipids could be loaded onto CD1 molecules for presentation to CD1 restricted lymphocytes (Schaible et al. 2000). Mtb exploits this pathway to gain access to DCs via DC-SIGN. In doing so they can impair DC function and down-regulate DC mediated responses (Geijtenbeek et al. 2003; Mihret, 2012).

NK cells form part of the innate immune response, and have been identified as inflammatory cytokine producing and cytotoxic lymphocytes and are largely known for their role in protection against viral infections and tumorigenesis (Sivori et al. 1997; Moretta et al. 2002), as well as controlling Mtb infection (Guerra et al. 2012). NK cells mediate their effector function indirectly by activating macrophages via IFN-γ and directly through the production of cytotoxic granules such as perforin, granzyme and granulysin (Liu et al. 2017). NK cells can also kill Mtb infected monocytes through direct contact and not via IFN-γ or cytotoxic granules (Brill et al. 2001). Additionally, it has been reported that NK cells can produce IL-22 and restrict intracellular Mtb growth by enhancing phagolysosomal fusion (Dhiman et al. 2003; Allen et al. 2015). Furthermore, IL-21 produced by T cells plays a role in the regulation of NK cell function and NK cells activated by IL-21 produce cytotoxic granules that lyse Mtb infected monocytes (Paidipally et al. 2018). NK cells are associated with early resistance against intracellular pathogens (Junqueira-kipnis et al. 2003). Mice infected with Mtb by aerosol, have an increased frequency of IFN-γ producing NK cells in their lungs (Junqueira–Kipnis et al. 2003). After depleting the NK cells, there was no difference in the lung bacterial burden, suggesting that removing NK cells do not necessarily have an impact in the early protective response to Mtb. Using cell-type deconvolution of transcriptomic data from several cohorts of different ages, genetic backgrounds, geographical locations and infection stages, an increase in the abundance of circulating NK cells in individuals with LTBI, with a corresponding decrease during active disease and higher levels upon clinical cure are features that are common to all cohorts (Chowdhury et al. 2018).

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Neutrophils play an important role in TB immunity, they are the first, and most abundant cell type to be recruited to the lung after Mtb infection (Korbel et al. 2008). The role of neutrophils during Mtb infection is conflicting, for example, antimicrobial effectors from neutrophil granules such as elastase, collagenase, and myeloperoxidase can mediate both anti-mycobacterial activity and immunopathology in human TB (Dallenga and Schaible, 2016; Sia et al. 2015). One mechanism which leads to immunopathology involves the ESAT-6 mediated induction of an intracellular calcium overload in neutrophils which is followed by necrosis of the cells and the formation of neutrophil extracellular traps (NETs) (Francis et al. 2014). Death of neutrophils results in NETs which contain DNA, myeloperoxides and metalloproteinases (Parker et al. 2012; Francis et al. 2014). A neutrophil-driven IFN-inducible gene profile, consisting of both IFN-y and type 1 IFN-α and -β signalling was reported to be a signature for TB disease and to be involved in the pathogenesis of TB (Berry et al. 2010). Absolute neutrophil counts are high in TB patients at diagnosis and reduce to levels compared to healthy controls at the end of TB treatment (Veenstra et al. 2006). In addition, this increase in blood neutrophils is associated with delayed sputum conversion (Martineau et al. 2011). Since the discovery of the IFN related gene signature, many investigated the role of type 1 IFN in innate immune responses during Mtb infection (Donovan et al. 2017). Type I IFN signatures in TB patients are associated with neutrophils and studies in mice with increased susceptibility to Mtb suggest that type I IFN signalling facilitates infection of permissive neutrophils resulting in damaging tissue pathology (Donovan et al. 2017). IFN-αR knock out in mice known to be susceptible to Mtb showed increased survival compared to wild type mice infected with Mtb *H37Rv* (Dorhoi et al. 2014). Furthermore, early depletion of neutrophils rescued TB-susceptible mice to levels observed in mice lacking IFN-αR1. It was concluded that type 1 IFN alters early innate events at the site of Mtb invasion leading to fatal immunopathology (Dorhoi et al. 2014). Despite the immunopathology aspect of neutrophils (Berry et al. 2010), they restrict Mtb growth *in vitro* (Korbel et al. 2008). Neutrophil peptides 1-3, cathelicidin LL 37 and lipocalin 2, have the ability to restrict Mtb growth and the depletion of neutrophils in whole blood results in poor Mtb control (Martineau et al. 2007). Loss of autophagy related gene 5 by neutrophils sensitizes mice to Mtb infection (Kimmey et al. 2015). Type 1 IFN controls *Mycobacterium bovis* and *Mycobacterium smegmatis* growth in infected mice (Kuchtey et al. 2006; Ruangkiattikul et al. 2017). The exact role of neutrophils during Mtb infection is controversial and this has been attributed to the difficulties of working with the cells. They are short lived cells, easily activated and cannot be cryopreserved (Lowe et al. 2012). There is thus an urgent need for further studies on the role of neutrophils during Mtb infection.

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5.2 Innate immunity in TB-T2D

A study conducted by Gomez and colleagues showed that there is a reduced association of Mtb with monocytes in T2D patients (Gomez et al. 2013), suggesting that the ability of the host cells to recognize Mtb is altered. The reduced association was likely due to the reduced expression of complement and Fc-gamma receptors (Restrepo et al. 2014). Initially, it was shown that alveolar macrophages (AMs) in TB-T2D patients are less activated compared to TB patients, which may contribute to susceptibility to Mtb infection (Wang et al. 1999; Lecube et al. 2011). TB patients have hypodense AMs which are also correlated to severity of disease, and activation. TB-T2D patients have low hypodense AMs suggestive of less activation (Wang et al. 1999). The level of activation was measured by the ability of cells to phagocytose *E.coli* bioparticles. Phagocytic capacity of AMs from TB-T2D is also lower compared to AMs from TB patients (Lecube et al. 2011). Furthermore, altered innate immunity was shown in TB-T2D using a mouse model (Vallerskog et al. 2011). Mice had reduced expression of chemokine CCL2 and CCL5, that stimulate migration of macrophages and DC to the lung (Vallerskog et al. 2011). The delay in macrophage recruitment results in delayed priming of the adaptive immune response that is necessary to restrict Mtb replication (Vallerskog et al. 2011).

In the lung, DCs are highly represented but if DCs cannot migrate from the lung to the draining lymph nodes, their ability to activate antigen specific T cells is compromised (Khader et al. 2006). T2D influences the phenotype and function of DCs in Mtb infected individuals and patients with TB (Kumar et al. 2015). The frequencies of DC are lower in Mtb infected individuals and TB patients with T2D compared to no T2D (Kumar et al. 2015). In addition, DCs were found to show a trend towards a negative correlation with hyperglycaemia during TB disease (Kumar et al. 2016). Kumar et al. investigated the influence of TB treatment on the frequency of DCs (kumar et al. 2016). TB patients without T2D did not show any difference in the frequency of DCs during TB treatment while TB-T2D showed a diminished DC frequency at baseline and at month two of TB treatment, which increased at month 6 (Kumar et al. 2016). The authors suggested that hyperglycaemia drives these alterations in the frequency of DCs in TB (Kumar et al. 2016).

TB-T2D patients have altered NK cell responses with elevated frequencies of TNF-α, IL-17A and IL-17F secreting NK cells compared to TB patients, following stimulation of whole blood with Mtb antigens (ESAT-6 and CFP-10) (Kumar et al. 2015). The frequency of NK cells expressing the degranulation molecule, CD107a, was diminished in TB-T2D compared to TB during Mtb antigen stimulation (Kumar et al. 2015). Since T2D alters the NK cell response to Mtb, it was suggested that this alteration contributes to the increase in disease severity and/or immune-mediated pathology observed in TB patients with T2D (Kumar et al. 2015). In whole blood from T2D patients and healthy individuals, the frequency of NK cells was not different

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between the groups (Guo et al 2012). The expression of natural killer group 2 (NKG2D) member was, however, positively correlated to body mass index and the CD107a produced by NK cells was higher in the T2D patients than controls. It was suggested that the activated NK cells may be involved in obesity dependent chronic inflammation associated with T2D pathogenesis (Guo et al. 2012).

As previously mentioned, neutrophils play an important role in TB immunity (Korbel et al. 2008). The role of neutrophils in recurrence and increased susceptibility to mycobacterial infection in T2D have been suggested before (Moutschen et al. 1992). TB-T2D have been associated with increased neutrophilic rich inflammation (Andrade et al. 2014; Prada-medina et al. 2017). There is a significantly higher number of neutrophils in blood of T2D patients, but they exhibit reduced capacity to phagocytose mycobacteria (Raposo-Garcia et al. 2017). Chemotaxis of neutrophils is lower in diabetic patients compared to healthy controls (Tater et al. 1987). Furthermore, activation of neutrophils and bactericidal activity was lower in neutrophils from diabetic patients compared to healthy individuals (Delamaire et al. 1997). In a streptozotocin (STZ) induced murine model of chronic diabetes (diabetes induced by STZ for more than three months) there was a higher Mtb burden in the lungs compared to normal non-diabetic mice (Martens et al. 2007), highlighting the inability of diabetic mice to control Mtb. Mycobactericidal activity of neutrophils did not differ between T2D patients and healthy individuals when their PBMCs were infected with Mtb (Raposo-Garcia et al. 2017)**.** Although most reports show impaired phagocytic activity, chemotaxis and activation of neutrophils in T2D compared to healthy individuals (Delamaire et al. 1997; Raposo-Garcia et al. 2017), contradicting results in the ability of neutrophils to kill bacteria exist. C3Heb/FeJ mice are highly susceptible to Mtb and form hypoxic, neutrophilic driven, necrotic granulomas upon infection with Mtb (Vilaplana et al. 2013). Treatment with ibuprofen for three to four weeks, after infection, improved both pathology and mycobacterial loads. After one week of ibuprofen treatment, fewer and smaller lung lesions with intra-alveolar neutrophils were observed in treated mice, whereas untreated mice had central caseous necrotic areas. Treated mice also had reduced pulmonary mycobacterial burden and extended survival (Vilaplana et al. 2013). This study shows that repurposing this widely used drug, suitable for children, ameliorates massive neutrophilic-associated inflammation upon Mtb infection (Dallenga and Schaible, 2016). The reported data shows that neutrophilic inflammation in T2D patients could also be targeted for host directed therapy (HDT) for TB treatment in TB-T2D patients (Prada-Medina et al. 2017).

6. Innate-like T cells

Innate-like T cells can rapidly produce cytokines after exposure to an antigen and are implicated in the defence against Mtb infection (Huang, 2016). They are activated by the

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major histocompatibility (MHC) class 1-like molecules (Huang, 2016). Innate-like T cells are mostly restricted by Cluster of differentiation 1 (CD1) and MHC-related protein 1 (MR1) (Huang, 2016). CD1 is restricted to invariant natural killer T (NKT) and MR1 is restricted to mucosal associated invariant T (MAIT) cells. Although innate-like T cells are known to be abundant in mucosal sites (Dusseaux et al. 2011; Kenna et al. 2003), MR1, CD1a and CD1c restricted T cells are also frequent in the periphery (Gold et al. 2010; de Lalla et al. 2011; Jong et al. 2014). The importance of innate-like T cells is due to their ability to express invariant T cell receptor α (TCRα) sequences (Porcelli et al. 1993).

6.1 Innate-like T cells in TB

NKT cells are predominantly $CD8^+$ (Ho et al. 2002; Gumperz et al. 2002) and express an invariant TCRVα24 chain paired with the TCRVβ11 chain, which enables the recognition of glycolipid antigens such as α-galactosylceramide (α-GalCer) on the MHC-1 like molecule CD1d (Godfrey et al. 2004). NKT cells express the surface receptor NK1.1, which is also expressed on NK cells (Tudhope et al. 2010). NKT cells are not cytotoxic (Smyth et al. 2002), even though they express perforin, Fas ligand (FASL) and NKG2D (Godfrey et al. 2004). Upon activation, NKT cells upregulate CD40L and activate monocytes, DCs, NK, T and B cells by producing IFN-y and IL-4, showing their importance in the transition from innate to adaptive immunity (Kaer, 2005; Cerundolo et al. 2009). NKT cells can function as either regulatory or effector cells in different diseases (Godfrey et al. 2004; Wermeling et al. 2010). The low frequency of NKT cells in human peripheral blood mononuclear cells (PBMCs) (between 0.05-0.92%) (Montoya et al. 2007), impacts the study of these cells in humans. The frequency of NKT cells can be expanded by alpha galactosaceramide and cultured for longer period with autologous irradiated mononuclear cells which can then change their surface receptors and may differ from unmanipulated resting NKT cells (Montoya et al. 2007).

In a murine Mtb infection model, treatment with αGalCer improved infection outcome by activating T cells through NKT cell activation, similarly to mice that were treated with standard TB chemotherapy (Sada-ovalle et al. 2010). In another study, co-culturing splenocytes from uninfected mice with Mtb infected macrophages, showed suppressed bacterial load due to NKT cell function (Sada-ovalle et al. 2008). NKT cells have been reported to play a protective role in the immune response of mice against Mtb, but not much is known about their role in human Mtb (Kee et al. 2012). To address this, PBMCs were isolated and were phenotypically defined using flow cytometry. Patients with TB disease had a lower frequency of NKT cells compared to individuals with LTBI (Kee et al. 2012; Montoya et al. 2008; Snyder-cappione et al. 2007; Sutherland et al. 2009). NKT cell frequencies are similar in individuals with LTBI and healthy controls, but higher than TB patients (Snyder-cappione et al. 2007; Sutherland et al. 2009). In contrast to these studies, Veenstra et al. showed that the frequency of NKT cells in

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TB patients was higher compared to healthy controls and correlated with a faster TB treatment response (Veenstra et al. 2006). When used in combination with NK cells they were identified as variables indicating the likelihood of culture conversion early during TB treatment (Veenstra et al. 2006). It was suggested that these cells hold promise to be considered as markers for TB treatment response (Veenstra et al. 2006).

MAIT cells are predominantly $CD8^+$, with a small subset being $CD4^+$ (Reantragoon et al. 2016). These cells express an invariant TCRVα7.2, C-type lectin-like receptor CD161 and dipeptidase CD26 and produce IFN-γ, granzyme B, granulysin and IL-17 (Gold and Lewinsohn, 2013; Sharma et al. 2015). MAIT cells are activated by riboflavin precursor metabolites in an MR1 dependent manner (Kjer-Nielsen et al. 2012;Gold and Lewinsohn, 2011). They can also be activated by cytokines in a MR1 independent manner owing to IL-18R, IL-12R and IFNR that are expressed on the MAIT cells (Le Bourhis et al. 2011;Ussher et al. 2015). MAIT cells upregulate granzyme B and perforin following activation and greatly enhance their killing ability of the infected cells (Dusseaux et al. 2011; Kurioka et al. 2014). In addition, MAIT cells have chemokine receptors, CCR2, CCR5, CCR6 and CXCR6 that allows them to reside in mucosal tissues (Dusseaux et al. 2011; Le Bourhis et al. 2011) (Figure 1.4). They also express transcription factors including RAR-related orphan receptor γt (RORγt), T-bet and pro-myelocytic leukemia zinc-finger (PLZF) at rest (Leeansyah et al. 2014; Walker et al. 2012) (Figure 1.4).

MAIT cells predominantly reside in the gut where their accumulation is dependent on MR1 expressing B cells and commensal flora (Martin et al. 2009). They also occur in the blood where 1-10% of the T cells are MAIT cells (Dusseaux et al. 2011). The frequency of MAIT cells in bacterial and viral infections, as well as in metabolic and autoimmune diseases vary (Billerbeck et al. 2010; Magalhaes et al. 2015; Loh et al. 2016; Petersone and Walker, 2017), implying that they play an important role in the regulation of immune responses in these diseases. Low frequencies of MAIT cells in the blood during TB disease is due to their migration to the site of disease (Gold et al. 2010). Wong et al. also demonstrated this after measuring MAIT cell frequencies in the broncho alveolar lavage fluid (BALF) and blood of TB patients and TB-HIV co-infected patients (Wong et al. 2012), higher frequencies of MAIT cells were found in the BALF than in the periphery. The addition of MAIT cells to BCG-infected macrophages resulted in the increased killing of the bacteria, demonstrating the protective role MAIT cells play during Mtb infection (Chua et al. 2012). MAIT cells not only assist in the control of bacterial infection, but also in the detection of bacterially infected cells (Gold et al. 2010; Le Bourhis et al, 2010). Cytokines such as IL-12, IL-18 produced by activated macrophages during Mtb infection can activate MAIT cells to release IFN-γ and TNF-α (Le Bourhis et al. 2011). Since MAIT cell frequencies are reduced during TB disease, it is

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suggested that these cells can be used as biomarkers to differentiate between LTBI and active TB. In addition, MAIT cell frequencies can be used to monitor TB treatment outcomes (Sharma et al. 2015).

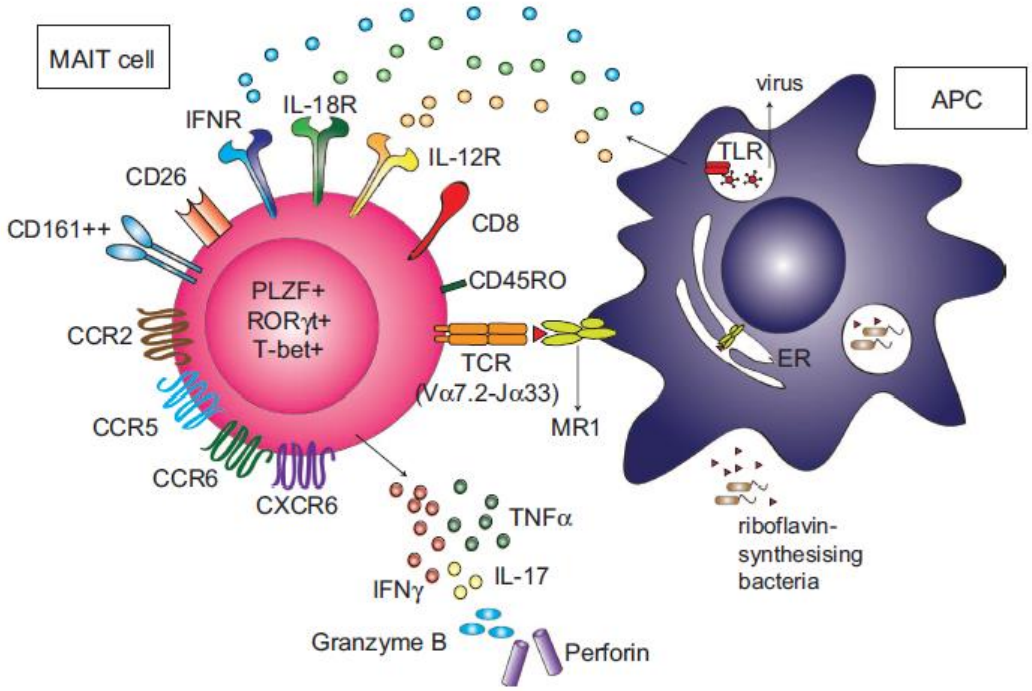

Figure 1.4: MAIT cell phenotype and different cellular activation pathways. Mature MAIT cells in peripheral blood express the chemokine receptors CCR2, CCR5, CCR6, CXCR6, the C-type lectin-like receptor CD161, the dipeptidase CD26 and a CD45RO+CCR7− effector memory phenotype, with the majority of human MAIT cells expressing the CD8 co-receptor. MAIT cells also express the transcription factors RAR-related orphan receptor γt (RORγt), T-bet and promyelocytic leukemia zinc-finger (PLZF) at rest. During bacterial infection, derivatives of the riboflavin biosynthesis pathway are presented by MR1 on the surface of APCs. Alternatively, viruses can also rapidly activate MAIT cells in an MR1-independent manner due to the induction of IL-18, IL-12 and IFNα. Activated MAIT cells express IFN-γ, TNF-α, granzyme B, perforin and IL-17 (Reproduced from (Kurioka et al. 2016)).

6.2 Innate-like T cells in TB and T2D

Little is known about the role of NKT cells in the pathogenesis of T2D. To address this, blood from T2D patients and healthy controls were investigated for activated and inhibitory NKT cells by using flow cytometry (Guo et al. 2012). There was no difference in the frequency of NKT cells in T2D patients compared to healthy individuals (Guo et al. 2012). NKT cell frequency was also examined in PBMCs and BALF of TB patients with and without T2D (Zhang et al. 2011). There were more NKT cells in blood and BALF from TB patients with T2D compared to TB without T2D (Zhang et al. 2011). Further analysis in PBMCs showed that NKT cells were higher in TB regardless of T2D status but lower in T2D patients with no TB and also in healthy controls (Zhang et al. 2011). This led to suggest that NKT cells measurements could be potential diagnostic marker for TB disease (Zhang et al. 2011).

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In a disease such as DM which is a risk factor for TB, the frequency of MAIT cells are altered (Magalhaes et al. 2015). MAIT cells were shown to be activated under changes in the make up of the gut microbiota, their activated form are associated with increased Th1 and Th17 cytokines (Andreone et al. 2018). MAIT cells exhibiting high levels of granzyme B as well as proinflammatory cytokines are thought to directly kill beta cells in humans with T1D (Rouxel et al. 2017), which could have an effect on the production of insulin. In obesity, however, MAIT cells were correlated to fasting insulin levels in children (Carolan et al. 2015) and negatively correlated to body mass index (BMI) in adults (Carolan et al 2015). Furthermore, bariatric surgery improved metabolic parameters such as BMI, HbA1c, leptin, adiponectin and was also associated with increased MAIT cell frequency (Magalhaes et al. 2015). There is however, no information on the association of MAIT cell frequencies with hormones like cortisol or DHEA which are known to have immunomodulatory effects (Buford et al. 2008; Dong et al. 2016). In end stage renal disease (ESRD), a complication of DM, MAIT cells are diminished (Juno et al. 2018). Individuals with LTBI suffering from ESRD have a 7-50 fold chance of developing TB (Hu et al. 2018; Mota et al. 2015). The MAIT cells in patients with ESRD have altered GM-CSF responses to microbial stimulation (Juno et al. 2018). Aging has also been associated with a reduction in MAIT cells and their ability to produce IFN-γ (Geest et al. 2018), in addition, T2D is also common in elderly people (Kirkman et al. 2012). Whether the alterations in MAIT cells in T2D patients are associated with the susceptibility of these patients to develop TB is not known. What we do know is that they are diminished and less activated in T2D as shown by the reduced expression of CD69 and CD25 as well as reduced IFN-γ and TNF-α production (Magalhaes et al. 2015). However, the exact role of MAIT cells in metabolic diseases is unclear. Furthermore, little is also known about MAIT cell frequencies and functions in TB-T2D patients.

7. Adaptive immunity

7.1 Adaptive immunity in TB

Adaptive immunity is an antigen specific response and has memory, to be able to mount an immune response during a second encounter with the same antigen. The ability of the host to recognize pathogens is important for the initiation of the adaptive immune response to an infection (Chackerian et al. 2002). The adaptive immune response is characterized by the activation of T ($CD4^+$ and $CD8^+$) and B lymphocytes. Infected innate cells, like macrophages and DCs, present antigens to the T lymphocytes (Dgeda et al. 2010). Upon recognizing the antigen, naïve T cells will differentiate into T cytotoxic lymphocytes (TCL ($CD8^+$)), T helper cells (Th1, Th2, Th17) and T regulatory (Treg) cells, to name a few. Th17 cells are known to secrete cytokines, such as IL-17, IL-21 and IL-22, which are responsible for the recruitment of monocytes and neutrophils to the site of infection (Dheda et al. 2010). Tregs inhibit the

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production of cytokines and T cell proliferation, and modulate Th1, Th2 and Th17 responses (Josten and Ottenhof, 2008). It is known that progressive TB disease is associated with low Th1 and high Th2 activity, whereas in close contacts (CCs) of TB patients there is a high Th1/Th2 ratio (Lienhardt et al. 2002). Patients who present with a favourable outcome at the end of TB treatment show a higher Th1/Th2 ratio as opposed to patients with poor clinical outcome (Lienhardt et al. 2002). Mtb infection induces IFN-y responses in both $CD4^+$ and $CD8^+$ T cells, which play a central role in the containment and killing of the bacterium (Kaufman and McMichael, 2005). The Th1 cells produce IFN-y, IL-2, TNF-α, IL-12, GM-CSF that incite the activation of Th1, $CD8^+$ CTL, macrophages and neutrophils to kill the invading pathogen (Dgeda et al. 2010). Once activated, $CD8^+$ cells secrete granulysin, granzyme and perforins, and directly kill the cells infected with Mtb (Ngai et al. 2007).

Cytokines such as IL-4, IL-5, IL-10 and IL-13 are produced by Th2 cells and activate B lymphocytes. B cells are responsible for antibody production and antigen presentation (Dheda et al. 2010). The presence of B cells and antibody (Ab)-responsive immune cells in TB granulomas supports their involvement in immune responses against Mtb infection (Tsai et al. 2006). The role of B cells have been studied in mice, where mice deficient in B cells showed higher splenic, lung and liver bacterial loads, six weeks after intravenous Mtb infection compared to mice without B cell deficiency. B cells can therefore exert beneficial effects on host responses to tuberculous infection (Vordermeier et al. 1996). In another study, B cell knock out mice were aerosol infected with a low dose of a clinical Mtb strain (CDC 1551) and compared to wild type mice (Bosio et al. 2000). B cell knock out (BKO) mice had less lesions in the lung and delayed dissemination of bacteria from lungs to peripheral organs. BKO mice in which naive B cells were reintroduced, but not mice given Mtb specific antibodies (before infection), developed pulmonary granulomas and dissemination patterns similar to wild type mice (Bosio et al. 2000). Mtb specific antibodies were obtained through cardiac puncture of mice that were infected with the Mtb CDC 1551 strain for 30 days, a period associated with the secretion of antibodies against a variety of mycobacterial antigens (Huygen et al. 1990). In this report, B cells were associated with increased cellular infiltration of the lungs by macrophages, neutrophils and $CD8^+$ T cells in wild type mice (Bosio et al. 2000). Although there is contradicting reports on the role of B cells in TB, their functions, such as antibody production and antigen presentation, show that they are an important immune cell type that requires further exploring.

7.2 Adaptive immunity in TB-T2D

TB-T2D is characterized by increased levels of Th1 (IFN-y, TNF-α, and IL-2), Th2 (IL-5, IL-10) and Th17 (IL17-A) cytokine responses (Kumar, et al. 2013). Stimulation of whole blood from TB-T2D patients with PPD, showed a higher Th1 cytokine profile but not Th2 cytokines

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(Restrepo et al. 2008). Th1 and Th17 cytokines positively correlate with HbA1c which means that patients with poorly controlled DM have higher levels of these cytokines (Kumar et al. 2013). These increased Th1 and Th17 mediated cellular responses are likely contributing to increased immune pathology in TB disease during T2D (Kumar et al. 2013). Recently, Wang et al. found that the proportion of Th2 and Th17 cells, but not Th1 cells, are significantly increased in TB patients with T2D after antigen stimulation of whole blood cells (Wang et al. 2018). These findings were confirmed in a study where PBMCs were stimulated with Mtb antigens and TB-T2D patients showed lower Th1/Th2 cytokine ratios, and a higher Th2 bias (Al-Attiyah and Mustafa, 2009). The decrease in Th1/Th2 ratio noted by Wang et al. in TB patients with T2D suggests there is an anti-inflammatory or Th2 bias in these patients that could contribute to the increased susceptibility seen in these patients (Wang et al. 2018). However in our cytokine analysis we found that TB-T2D patients had higher Th2 cytokines (IL-4, IL-5, IL-10 and IL-13) (Manuscript in preparation).

High expression of IL-22 is beneficial to the host in many infectious and inflammatory disorders, and if produced in excess can cause immunopathology (Rutz et al. 2013). In TB-T2D, this cytokine was reported to be expressed at lower levels compared to TB patients (Kumar et al. 2013). In addition, TB-T2D has also been associated with increased systemic levels of pro-inflammatory (IL-1β, IL-6 and IL-18) and anti-inflammatory (IL-10) cytokines (Kumar et al. 2013). Varying results have been reported with some showing no difference in Th1 cytokines in TB patients with and without T2D (Gan et al. 2014; Stalenhoef et al. 2008).

The influence of T2D on immune cells were investigated by staining whole blood with monoclonal antibodies for various immune cell types using flow cytometry (Kumar et al. 2015). TB-T2D patients have lower frequencies of effector memory $CD8^+$ T cells and higher frequencies of central memory T cells compared to TB patients. Classical memory B cells were higher in TB-T2D compared to TB patients (Kumar et al. 2015). TB-T2D patients also have a higher frequency of $CD4^+$ T cells producing Th1 cytokines compared to TB patients after stimulation of whole blood with Mtb antigens (Kumaret al. 2013). This hyperactive antigen specific T cell response in TB-T2D that is significantly higher than responses in TB patients without T2D may be associated with increased lung pathology in TB patients with T2D (Kumar and Babu, 2017). $CD8^+$ T cells in TB-T2D patients are characterized by diminished granzyme B, perforin and CD107a production compared to TB without T2D, which are important for killing of infected cells (Kumar et al. 2015).

8. Gene expression analysis in TB

Several studies have successfully shown that blood gene transcripts are capable of differentiating individuals with active TB from those with Mtb infection. The use of biomarkers

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obtained from blood transcripts is fairly new and research in this field is increasing due to its contribution in identifying transcriptional signatures associated with diagnosing TB and TB treatment responses (von Both et al. 2015; Thompson et al. 2017). Transcriptional profiling of whole blood RNA revealed a 16-gene signature that predicts progression from LTBI to active TB diseases (Zak et al. 2016). This signature predicted progression to active TB, 12 months prior to diagnosis of TB disease (Zak et al. 2016). Following this study, it was thought that 16 genes are too many to incorporate in a simple point of care test, and was further optimized to reduce the number of genes. A four-gene signature was identified that predicts which patients with LTBI will develop active TB two years prior to diagnosis (Suliman et al. 2018; Thompson et al. 2018). Gene expression studies in serum exosomes (Lv et al. 2017), specific immune cells such as DCs (Blischak et al. 2017) and PBMCs (Serrano et al. 2016) also identified gene profiles able to predict susceptibility to TB disease. The risk of TB progression in LTBI with HIV and T2D is much higher than compared to otherwise healthy LTBI positive individuals (Jeon and Murray, 2008; Murray et al. 2014). The question is whether the identified signatures are equally applicable to LTBI positive individuals with other diseases. For example, the signature identified by Zak et al. (2016) was in HIV negative individuals and is there a need to test the signature in HIV positive and T2D cohorts, because these groups are at greater risk of TB. To address this, Dawany et al. reported that the 393-gene signature from Berry et al. (2010), could not identify TB in patients co-infected with HIV (Dawany et al. 2014). However, a transcriptional profile identified by Prada-Medina et al. in patients with TB-T2D shared elements with the profiles in TB patients without T2D (Prada-Medina et al. 2017).

In South Africa, preventative treatment is not given to LTBI positive individuals, because it would be too expensive and ineffective due to the high chance of re-infection in a TB endemic country. These signatures are therefore useful and can potentially be used to identify individuals with LTBI who are at increased risk of developing active TB and who can potentially be given prophylactic treatment in an attempt to reduce the TB burden (Cohn et al. 2000). In a whole blood transcriptomic study, Cliff et al. identified a transcriptional signature which correlated with the extent of TB disease in the lung as determined by radiography and concluded that immune responses in the blood can reflect the local reaction to Mtb in the lung (Cliff et al. 2015). Not only do blood transcript signatures identify individuals at risk of TB development, but can also inform of the severity of the disease in the lung. Thompson et al. further identified a five-gene signature that predicts TB treatment outcomes which correlated with the inflammatory state in the lungs of TB patients as measured by positron emission tomography–computed tomography (PET-CT) (Thompson et al. 2017). Bio-signatures to determine relapse and which can be applied to different ethnic groups and co-morbidities, including T2D are needed.

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9. Immune and endocrine interactions

Cytokines produced by activated immune cells feed back to the central nervous system and induce neuroendocrine responses that, in turn, can modulate immune responses to infections (Webster et al. 2002). Chemical messengers produced by the immune cells facilitate the communication between the immune and neuro-endocrine system and also the immune and central nervous system (CNS) (Dantzer et al. 1998; Bottasso et al. 2012). Chronic, low-level inflammation that occurs during T2D travels to the brain and activate the hypothalamic-pituitary-adrenal (HPA) axis, and the sympathetic system (Bottaso et al. 2012; Bouzaki and Zierath, 2007; Ottaviani and Francheschi, 1996; Chrousos, 2000). Activation of the HPA axis results in the production of corticotropin-releasing hormone (CRH) and vasopressin (VP), which travels via the portal vein to the pituitary gland and activate corticotropic cells, which subsequently produce adrenocorticotropic hormone (ACTH) (Antoni, 1986; Aguilera, 1994; Aguilera, 2012). ACTH travels to the adrenal gland which has two major zones; the cortex and the medulla, which will produce glucocorticoids (GCs) and catecholamines (CA), respectively (Rhen and Cidlowski, 2005; Aguilera, 2012). GCs inhibit Th1 responses by favouring Th2 cytokine responses (Howard and Zwilling, 1999). Chronic stress, for example, leads to the activation of the sympathetic system and results in the upregulation of the CAs, adrenaline and noradrenaline (Figure 1.5) (Webster and Glaser, 2008; Tian et al. 2014). GCs and CAs bind to their receptors on immune cell and inhibit the secretion of pro-inflammatory cytokines such as IL-1β, IL-6, TNF-α and IFN-y, and enhances the production of anti-inflammatory cytokines such as IL-4, IL-13 and IL-10 (Figure 1.5) (Elenkov and Chrousos, 1999; Tracey 2002). In addition, GCs will inhibit transcription factors such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB), activator protein 1 (AP-1), Janus kinase/signal transducers and activators of transcription (JAK-STAT), mitogen activated kinases, signal transducer and activator of transcription 3, which may in turn inhibit pro-inflammatory cytokine production (Tracey 2002) (Figure 1.5).

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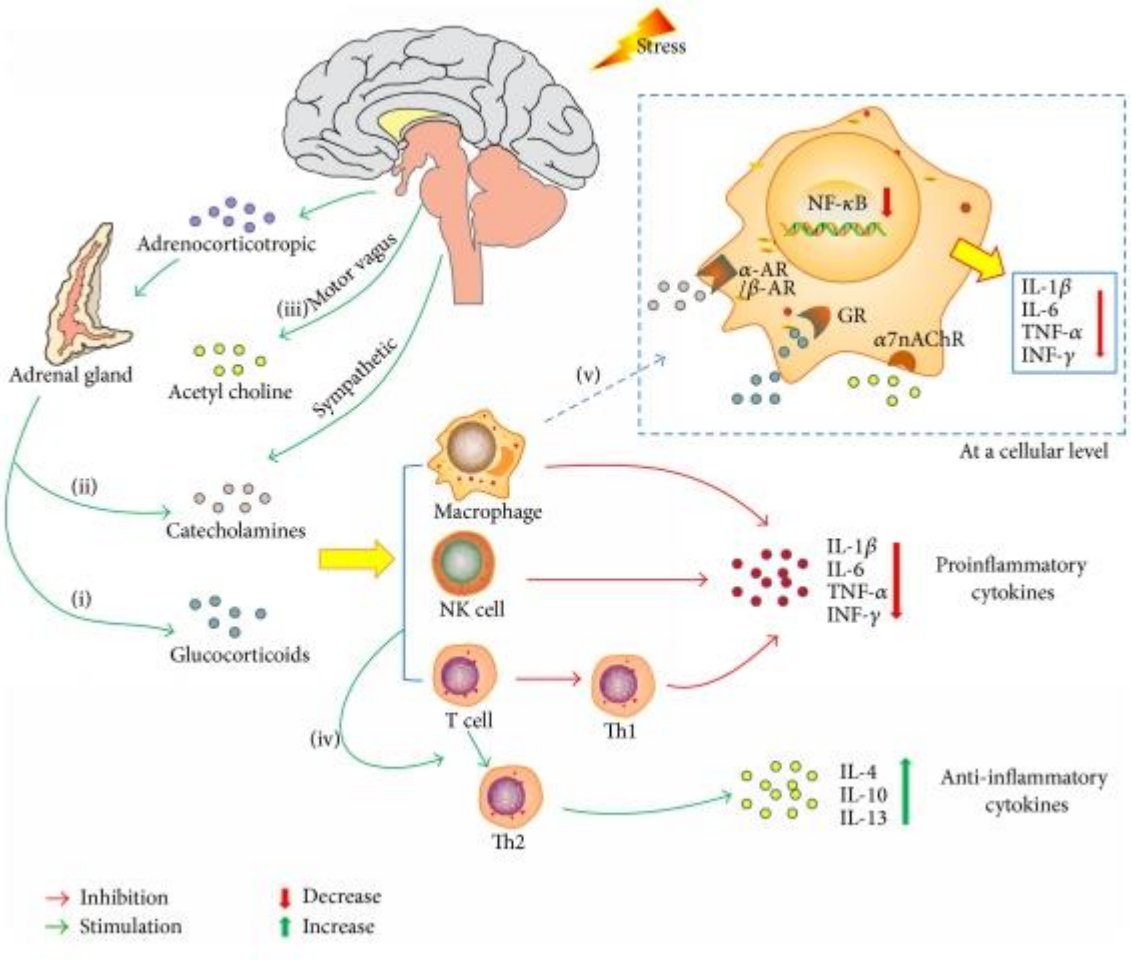

Figure 1.5: The activation of the HPA axis, sympathetic and immune system. Stress activates the HPA and Sympathomedullary axis which secrete (i) GC and (ii) CA hormones respectively, the hormones act on receptors on the surface or cytoplasm of the immune cells. (iii) Motor vagus secretes CAs, which in the end result in decreased pro-inflammatory cytokines while increasing anti-inflammatory responses. (iv) There is a shift to Th2 opposed to Th1 responses. (v) In the cell, the hormones inhibit inflammation related pathways including NF-kB and further inhibit pro-inflammatory responses (Adapted from (Tian et al. 2014)).

Serotonin, produced by nerve cells also has immune modulatory properties. Serotonin is stored in platelets and is released during platelet activation induced upon inflammation (Herr et al. 2017). The immunomodulatory effects of serotonin are mediated through binding to the serotonin receptors expressed on immune cells (Mossner and Lesch, 1998). Binding of serotonin to the 5 hydroxytryptamine-1 (5HT-1) receptor on monocytes, reduced the ability of the monocytes to suppress NK cell functions (Hellstrand and Hermodsson, 1993). In addition, the effect of serotonin on IFN-y-induced phagocytosis varies according to the concentration of IFN-y to which the macrophages are exposed (Sternberg et al. 1987). At low concentrations of IFN-y, serotonin augments phagocytosis, whereas at high concentrations of IFN-y, serotonin suppresses phagocytosis (Sternberg et al. 1987). Serotonin induces the production of various cytokines in monocytes, macrophages, DCs (IL-1β, IL-6, IL-8, IL-12p40 and IFN-γ)

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and lymphocyte (IL-2, IL-16 and IFN-γ), but supresses TNF-α production in monocytes, macrophages and DCs (Figure 1.6) (Duerschmied et al. 2014) supporting the notion of the bidirectional communication between central nervous and immune system.

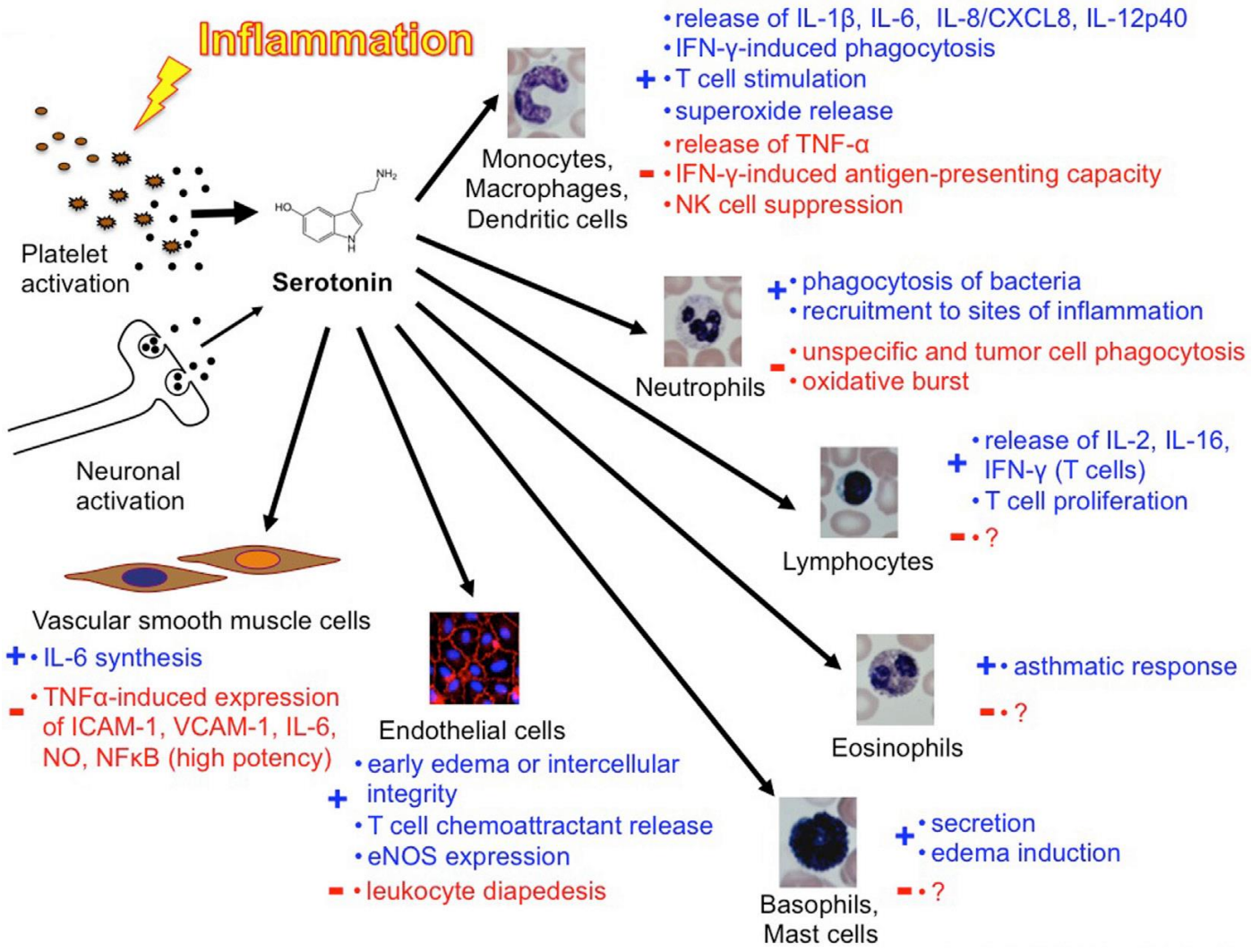

Figure 1.6: Immunomodulatory properties of serotonin. Blue - activated or upregulated, red - inhibited or reduced, question mark - unknown effect (Duerschmied et al. 2014).

9.1 Immunendocrine changes during TB

The pituitary gland produces ACTH (HPA axis), luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (HPG axis) as well as thyroid-stimulating hormone (TSH) (HPT axis) resulting in the production of cortisol by the adrenal gland, oestrogen and testosterone by the gonads as well as triiodothyronine and thyroxine by the thyroid gland. Activation of the HPA axis influences the behaviour of the immune system via GCs and dehydroepiandrosterone (DHEA), which counteracts the GC effects (Webster et al. 2002; Bottasso et al. 2007). GCs can both inhibit or stimulate the innate and adaptive immune responses (Webster et al. 2002). Del Rey et al. measured IL-10, IFN-y and IL-6 and several

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hormones of the pituitary, adrenal, gonadal and thyroid glands in male patients with newly diagnosed TB and compared them to healthy controls (del-Rey et al. 2007). TB patients had increased plasma concentrations of the above mentioned cytokines and decreased concentrations of testosterone and DHEA (del-Rey et al. 2007). Growth hormone (GH) concentrations were higher in TB patients, higher concentrations were seen for cortisol, estradiol, thyroid hormones and no differences were observed in insulin like growth factor 1 concentrations (del Rey et al. 2007). Immune responses during TB therefore affect endocrine functions (Turnbull and Rivier, 1999; Van den Berghe 2003). Since GCs favour Th2 responses, elevated concentrations of GCs are not favourable for the development of the effective cell mediated response against Mtb (Bottasso et al. 2013). Supernatants from cultures of Mtb stimulated PBMCs of TB patients have been shown to inhibit DHEA secretion by a human adrenal cell line (del-Rey et al. 2007). Chronic persistence of decreased DHEA concentrations and of increased cortisol/DHEA ratios have harmful consequences for the host due to uncontrolled inflammatory processes that may affect the control of tissue damage and the development of protective immune responses (Imrich, 2002). TB patients have higher cortisol, estradiol, prolactin, GH, thyroid hormone and dopamine concentrations, as well as lower adrenaline and noradrenaline concentrations when compared to healthy controls (del Rey et al. 2007; Opolot et al. 2015). TB patients also have higher IFN-y, TNF-α, C-reactive protein (CRP), IL-1β, IL-6 and IL-10 levels compared to healthy controls (del Rey et al. 2007; Opolot et al. 2015). Kleynhans et al. showed that concentrations and treatment responses of various hormones were different in patients with different TB treatment outcomes (Kleynhans et al. 2017). Cortisol concentrations were higher in TB disease and decreased during treatment and remained lower in the cured group while it was higher in individuals who failed TB treatment (Kleynhans et al. 2017). Cortisol concentrations in patients with a failed TB treatment outcome correlated with immune markers such as CRP and serum amyloid protein –A (SAP-A) and interleukin 2 receptor subunit alpha (IL-2Rα) at baseline, matrix metalloproteinase 9 at week four of TB treatment and interferon inducible protein-10 (IP-10) at the end of treatment (Kleynhans et al. 2017). Increased inflammatory marker levels during TB results in increased cortisol concentrations as part of a feedback mechanism to prevent excessive inflammatory responses and immunopathology. These data provide evidence that there exists a relation between endocrine and immune systems during TB and the interaction between these systems need to be studied further and can potentially be used as biomarkers for TB disease progression, severity and even treatment responses. There is a dysregulation of the HPA, HPG and HPT axis, and the production of pituitary hormones during severe TB disease, and such dysregulation might be responsible for the aggravation of the disease (Bottasso et al. 2007).

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9.2 Immune-endocrine changes in TB-T2D

T2D is a known risk factor for TB and studies have shown that both diseases have altered endocrine responses, which are likely to play a role in certain immuno-endocrine-metabolic associated disorders (Fernandez et al. 2016). As mentioned previously, endocrine alterations can contribute to TB disease outcomes (Bottasso et al. 2013). The chronic inflammation contributes to the activation of the HPA axis and increases the severity of infections or lead to susceptibility to such infections through the suppressive ability rendered by GCs (Besedovsky and del-Rey, 1996; Bottasso et al. 2013). The occurrence of both TB and T2D may present a particular trend of immune and endocrine activation that is worth investigating in order to understand the association between the two diseases (Fernandez et al. 2016). In light of these, Fernandez and colleagues analysed blood levels of cytokines, hormones and immune responses to mycobacteria in TB-T2D patients. It was found that TB patients had higher concentrations of cytokines compared to healthy controls whereas TB-T2D patients had even higher IL-6 and IFN-y compared to TB patients (Fernandez et al. 2016). IL-10 levels remained the same between TB and TB-T2D but lower in healthy subjects (Fernandez et al. 2016). In the same study, the concentration of cortisol was found to be higher in individuals with TB-T2D (Fernandez et al. 2016). Bottasso et al. showed that higher cortisol is associated with TB severity (Bottasso et al. 2013) and TB-T2D patients were previously reported to have more severe TB disease than patients with only TB (Baker et al. 2012; Gil-santana et al. 2016). Estradiol levels were high in patients with TB and significantly higher in patients with TB-T2D (Fernandez et al. 2016). Estradiol and cortisol were positively associated with IFN-y and IL-6 (Fernandez et al. 2016). The adverse immune-endocrine profile observed in TB patients is more pronounced during TB concomitant with T2D characterised by more intense immune and endocrine inflammatory reactions, which shows the detrimental effects of T2D on Mtb infection (Fernandez et al. 2016).

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10. Hypothesis, Aims and Objectives

Central hypothesis

We hypothesize that T2D causes a systemic dysregulation of the immune-endocrine networks which is associated with distinct blood bio-signatures and impaired monocytes and PBMCs function.

Aim 1:

The first aim of this project was to identify differentially expressed immune genes in unstimulated and Mtb-antigen stimulated whole blood of latently infected close contacts of TB patients as well as active TB patients with and without T2D.

Specific objectives were to:

1.1 Characterize messenger ribonucleic acid (mRNA) transcript signatures in unstimulated (nil) and Mtb-antigen stimulated whole blood collected from QFT tubes of individuals with LTBI with and without T2D.

1.2 Characterize the mRNA transcript signatures in unstimulated (nil) and Mtb-antigen stimulated whole blood collected from QFN tubes of TB patients with and without T2D at baseline and month 6.

Aim 2:

To investigate immune and endocrine changes in the serum and plasma of individuals with LTBI as well as TB patients with and without T2D.

Specific objectives were to:

2.1 Characterize cytokine and hormone levels in individuals with LTBI and TB patients with and without T2D using luminex and ELISA.

2.2 Determine whether any associations exist between cytokine and endocrine signatures with *in vitro* Mtb killing.

2.3 Determine whether any associations exist between glycaemic control and *in vitro* Mtb killing.

Aim 3:

To determine the frequency of MAIT cells in TB patients with and without T2D.

Specific objectives were to:

3.1 Determine the frequency of the MAIT cell population in TB and TB-T2D patients using flow cytometry.

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CHAPTER 2

Materials and methods

2.1.1 Informed consent and ethical clearance

This study was part of the ALERT and TANDEM studies. Ethical approval was obtained for the ALERT Study (Altered Immune-endocrine Axis in Type 2 diabetes and Tuberculosis risk:which was aimed at better understanding of the link between TB and T2D, to identify individuals at increased risk for TB progression) and the TANDEM study (Concurrent Tuberculosis and Diabetes: aimed at improving Care through bi-directional Screening and Unravelling the Causal Link through Study of Genetic Susceptibility Factors) from the Human Research Ethics Committee of the University of Stellenbosch (N13/05/064, TANDEM and N13/05/064A, ALERT). The studies were conducted according to the Helsinki Declaration and International Conference on Harmonization guidelines (WMA, 2001). Written informed consent was obtained from all study participants.

2.1.2 Study participants and sample collection

Individuals with LTBI with and without T2D were screened and enrolled as part of the NIH funded ALERT study. Individuals with LTBI were defined as individuals sharing at least five hours per week in a house or closed space with a confirmed pulmonary TB case, test positive for Quantiferon IFN-γ assay and negative for active TB (discussed in II below). TB patients with and without T2D were enrolled in the EU FP7 funded TANDEM study, before TB treatment (baseline, BL), after completion of intensive phase treatment at month 2 (M2) and at the end of TB treatment (M6). TB patients were recruited at six TB clinics, Fisantekraal, Ravensmead, Uitsig, Adriaanse, Elsies River and Durbanville, in Cape Town, Western Cape, South Africa. TB patients gave permission for study personnel to visit their homes and to invite their family members and close friends to take part in the study.

i. ALERT participants

Forty CCs of TB patients who tested positive for LTBI on a Quantiferon TB-Gold in-tube (QFT) assay were included in this study. All the ALERT participants were fasted and the specimens collected before 09:00AM to control for circadian rhythm. At enrolment, clinical parameters such as insulin, cortisol, lipid profiles, complete blood count (CBCs) and anthropometric measurements were determined. T2D status was diagnosed based on a FBG and HbA1c (American Diabetes association, 2010). Patients were classified as LTBI without T2D (LTBI-noT2D; n = 10; HbA1c < 5.6 %, FBG ≤ 100 mg/dL (5.6 mmol/L)), LTBI with preT2D (LTBI-PreT2D; n = 10; HbA1c 5.7-6.49 %, FBG 100-125 mg/dL (5.6-6.9 mmol/L)), LTBI with T2D (LTBI-T2D; n = 10; HbA1c 6.5-7.9 %, FBG ≥ 126 mg/dL (7 mmol/L)) and LTBI with poorly controlled T2D (LTBI-pT2D; n = 10; HbA1c ≥ 8 %, FBG ≥ 183 mg/dL (10.2 mmol/L)).

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ii. Inclusion and exclusion criteria for ALERT

The inclusion criteria were LTBI (based on positive QFT assay) and the age of 30 to 65 years. Individuals were excluded if they were HIV positive, pregnant or breast-feeding, if they had active TB (confirmed by positive GeneXpert MTB/RIF (Cepheid, California, USA), sputum culture or abnormal chest X-ray (CXR), type 1 diabetes, and if they used steroids or any other medication that affects immunity (i.e. non-steroidal anti-inflammatories).

iii. TANDEM participants

HbA1c was measured at BL and at M6 for TB patients. TB patients were classified as TB only (n = 9), TB with transient hyperglycaemia (TB-THG; n = 5) and TB with T2D (TB-T2D; n = 7) using HbA1c cut-offs as follow: HbA1c <5.6 % for TB, HbA1c ≥ 6.5 % for TB-THG at BL but normal at M6, and HbA1c ≥ 6.5 % for TB-T2D at BL and M6. Four of the five TB-THG patients had a family history of diabetes, but were not taking diabetes medication during the course of the study. All TB-T2D patients were on standard TB treatment (Isoniazid (INH), RIF, Ethambutol (EMB) and Pyrazinamide (PZA) for the first two months, and INH and RIF for the following four months) as recommended by the South African National Tuberculosis program, and all seven were on T2D medication (Insulin and/or metformin). Twelve uninfected HCs and 13 T2D patients without TB were included as controls. All the TANDEM participants were between the ages of 30 and 60 years and were age and gender matched.

iv. Inclusion and exclusion criteria for TANDEM

The inclusion criteria were TB disease confirmed by abnormal CXRs, positive GeneXpert MTB/RIF (Cepheid) and/or positive MGIT (BD BACTEC MGIT 960 system, BD, New Jersey, USA) culture. The TB patients received conventional TB therapy (described above). Participants were excluded if they were pregnant, HIV positive, had drug resistant Mtb and were using immunosuppressive drugs. All TB, TB-THG and TB-T2D patients used for this study adhered to and completed more than 80 % of their TB treatment and all were successfully cured at the end of treatment.

2.1.3 Sample processing for ALERT study

Blood collected in Sodium heparin (NaHep; BD), serum (Geiner bio-one, Kremsmunster, Austria), EDTA (Geiner bio-one) and Lithium heparin tubes (LiHep; BD)) and sputum were collected from all study participants at BL (ALERT and TANDEM), M2 and M6 of TB treatment (TANDEM).

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i. Plasma and serum isolation

EDTA (1x 6 mL) and serum (2x 6 mL) blood tubes were spun down at 12 000 xg (Eppendorf centrifuge 5702, Merck-Millipore, Massachusetts, USA) for 10 minutes and the plasma and serum aliquoted and stored at -80°C for further use.

ii. Quantiferon TB-gold in tube (QFT) processing

One mL of blood, collected in a LiHep tube (1x 4 mL), was transferred to each of the three tubes of the QFT assay (Qiagen, Hilden, Germany). The nil tube contains no additives and serves to adjust for background IFN-y production, the TB-Ag tube is coated with an antigen cocktail including ESAT 6, CFP-10 and TB 7.7 specific for Mtb and the mitogen tube contains phytohaemogglutin-P (PHA) which is a positive control. After adding the blood, the tubes were inverted ten times and incubated at 37 °C for 16-22 hours. Blood was transferred from the QFT tubes into a 2 mL cryogenic vials (Sigma-Aldrich, Missouri, USA) and centrifuged for 15 minutes at 15 000 xg. The supernatants were stored at -80 °C and the pellet stored in RNA*Later* (Thermo Fisher scientific, Virginia, USA) at 4 °C overnight, before being transferred to -80 °C. The preserved QFT pellets were used for RNA extractions and the supernatants used in an ELISA to measure IFN-y concentrations.

iii. Peripheral blood mononuclear cells (PBMCs) isolation

Blood collected in NaHep tubes (6x 9 mL) was used for PBMC isolations by density gradient centrifugation. Briefly, blood were transferred to 50 mL falcon tubes (Sigma-Aldrich) and centrifuged at 800 xg for 12 minutes at 20 °C (acceleration=9; brake=0). The plasma was transferred to a 15 mL falcon tube (Corning Science, Newyork, USA) and stored on ice. Care was taken not to disturb the white blood cell interphase. The remaining plasma, white blood cell interphase and top part of the red blood cell layer were transferred to a new 50 mL falcon tube (Corning Science) and mixed. PBS containing 1 mM EDTA (Sigma-Aldrich) and 1 % Human serum albumin (SEH) were added to the cells (up to 35 mL mark), to replenish the plasma, and mixed by pulse vortexing. The cells were layered onto 15 mL Ficoll-Paque-Plus (GE HealthCare Bio-science, Uppsala, Sweden) and centrifuged at 600 xg for 30 minutes at 20°C (brake=0; acceleration=0)**.** Plasma, including SEH, was removed except for 5-10 mL above the buffy coat. The buffy coat was transferred to a 50 mL tube and washed with cold RPMI-HEPES medium (Sigma-Aldrich). Cells were centrifuged at 4°C at 600 xg for four minutes (acceleration=9; brake=0) after which the supernatant was discarded. Cells were washed for a second time using RPMI-HEPES and spun down at 4°C at 150 xg for eight minutes (acceleration=9; brake=0) to remove excess platelets. The supernatant was discarded and the pellet resuspended in 5 mL of RPMI (Biowest, Nuaillé, France)

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supplemented with 2 mM glutamine (GLUT; Sigma-Aldrich) and 100U/mL penicillin (PEN; Sigma-Aldrich) (RPMI+GLUT+PEN).

iv. Counting of PBMCs and overnight culturing:

Ten µl of the cell suspension were added to 90 µl of 10 % Trypan blue (in PBS) and counted using a Countess Cell counter (Invitrogen, California, USA). The total cell count, live cell count, dead cell count and viability (%) were reported and the live cell count used to determine the number of cells needed for the downstream assays. Six hundred thousand cells were cultured overnight in RPMI+GLUT+PEN medium containing 20 % autologous plasma in 2 mL sterile screw cap tubes (Scientific specialists SSIBIO USA), in a final volume of 600 µl at 37 °C and 5 % CO_2.

v. Monocytes (MNs) isolation

MNs were isolated from PBMCs using the Miltenyi Pan Monocyte isolation kit and LS MACS separation columns (Miltenyi biotec, Bergisch Gladbach, Germany). PBMCs were spun down at 300 xg for 10 minutes at 4 °C and the supernatant discarded. FcR blocking reagent and biotinylated antibody (Ab) cocktail were added and the cells incubated for five minutes at 4 °C. MACS buffer was added as well as anti-biotin microbeads, the cells were again incubated for seven minutes at 4°C. During the incubation step, the columns were primed with MACS rinsing buffer (Miltenyi biotec) and the volume of the cell suspsension adjusted to a total volume of 500 µl using the MACS rinsing buffer. The cell suspension was applied to the column and the flow-through containing the monocytes collected in a 15 mL tube. The column was washed three times with 3 mL rinsing buffer. The cells collected as part of the flow-through was centrifuged at 300 xg for seven minutes at 4 °C, the supernatant discarded and the pellet resuspended in 1 mL RPMI+GLUT+PEN media. The MNs were counted, as mentioned above, and 130 000 cells/well were seeded in poly-D-lysine coated flat bottom plates (Corning Sciences, USA) in a final volume of 200 µL and incubated overnight at 37 °C and 5 % CO_2.

Purity checks were performed for the first participants and we confirmed the purity to be > 90 % with all of the isolations. Due to the limited number of cells, we did not do it for all participants. Purity check was performed by staining a fraction of the isolated MNs using monoclonal antibodies anti-human CD14 and anti-human CD16. The MNs were centrifuged at 400 xg for five minutes and the media was discarded. MNs were incubated with the antibodies for 30 minutes at 4°C. After washing with FACS buffer, MNs were fixed with 4 % formalin for 15 minutes in the dark. Formalin was washed off with 1 mL FACS buffer and the cells resuspended in 100 µl FACS buffer prior to acquisition. Stained cells were kept at 4°C until they were acquired on the FACSCanto II flow cytometry instrument (BD).

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vi. PBMCs and MNs infection with Mtb *H37Rv* to determine Mtb uptake and killing

After overnight culturing, the PBMCs were centrifuged at 400 xg for five minutes, the supernatant discarded and the cells washed with 600µl RPMI-HEPEs. MNs adhered to the poly-D lysine coated flat bottom plates, therefore no centrifugation was required before aspirating the media and washing the cells with 200 µl RPMI-HEPES. PBMCs and MNs were infected for two hours with Mtb *H37Rv* at an MOI of 3.25:1 and 1:1 respectively, washed and further incubated for one day and three days (for MNs) or six days (for PBMCs) in RPMI+GLUT media supplemented with 20 % autologous plasma at 37 °C and 5 % CO_2. Culture supernatants collected from PBMC (after two hours, day one, day three and day six) and MNs (after two hours, day one and day three) were filter sterilized using Millex 4 mm syringe filters (Merck-Millipore) and stored at -80°C until cytokine profiling.

At each of the time points, PBMCs and MNs were washed and lysed using 0.05 % SDS for five minutes. The SDS was diluted out by adding an equal volume (300µL) of Middlebrook 7H9 broth (BD). The lysates were serially diluted and plated on 7H11 Middlebrook (BD) plates. The plates were monitored weekly for contamination and the colony forming units (CFU) enumerated after three weeks. Additional PBMCs were removed from the tubes using 2 mM EDTA (Sigma-Aldrich) and stored in RNA*Later* (Invitrogen) at 4 °C overnight and transferred to -80 °C the following day until RNA was isolated.

Some MNs were treated with 100 µl of Cetyltrimethyl ammonium bromide (CTAB; Sigma-Aldrich) for 10 minutes to lyse the cells. Nuclei were then counted using a haemocytometer to determine the number of viable cells.

2.1.4 Sample processing for the TANDEM study

Serum, plasma, whole blood (stored in RNA*Later*) and cryopreserved PBMCs were previously collected and stored as part of the TANDEM study and analysed as mentioned below.

2.1.5 RNA extraction study

RNA was extracted from the QFT assay cell pellets for both the ALERT and TANDEM study. The pellet, which was stored in RNA*Later* (as mentioned above), was thawed and total RNA extracted using the Ribopure Ambion RNA isolation kit (Life Technologies, Carlifornia, USA). The extraction was done according to the manufacturer's instruction. Briefly, samples were thawed and spun down at 14 000 xg (Eppendorf centrifuge 5415C, Merck-Millipore) for one minute. The supernatants were discarded and lysis buffer added to the pellets. Sodium acetate solution was added and the samples vortexed to ensure all the cells were lysed. Chloroform (350 µl) was added to the cell lysates and the samples vortexed to remove DNA and proteins, followed by five minutes incubation at RT. The mixture was centrifuged for a minute at 14 000 xg and the aqueous phase transferred to a new microcentrifuge tube. Absolute molecular

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grade ethanol (600 µl) was added to the aqueous phase and the samples vortexed. The samples were transferred to a column, centrifuged and the flow through discarded. The column was washed with buffer 1 and again with buffer 2/3 and centrifuged for 10 seconds at 14 000 xg after each wash step. The RNA was eluted in a total volume of 100 µl elution buffer. The eluted RNA was treated with 8 U/ µl DNase I, which is included in the Ribopure Ambion isolation kit (Life Technologies), for 30 minutes, to remove DNA. DNase activity was halted by adding the inactivation reagent and incubating the samples for two minutes at RT. After centrifuging the sample, the supernatant was collected into a new tube and quantified (see below). RNA samples were divided into 20 µl aliquots, to avoid multiple freeze-thaw cycles, and stored at -80 °C.

2.1.6 RNA quantification, gene expression assay and data analysis

Total RNA was quantified using the Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific) and the RNA Qubit kit (Life technologies, Oregon, USA). Samples with a concentration of ≥ 20 ng/µl and a 260/280 and 260/230 ratio of ≥ 1.7 were included in the NanoString analysis. Eighty-four TANDEM and 80 ALERT samples were shipped to NanoString Technologies in Seattle Washington through their Cape Town supplier, Anatech, to determine the gene expression of 594 genes included in the nCounter GX Human Immunology kit V2 (supplementary Table 1). The nCounter analysis platform from NanoString is a direct digital detection assay of individual target molecules and does not require any amplification or reverse transcription step. Briefly, 100 ng of each RNA sample was added to the hybridization buffer containing the Codeset and incubated at 65 °C for 16 hours. The CodeSet consists of reporter and capture probes that hybridize to the target sequences of interest, forming a tripartite complex. The complexes were purified and immobilized onto the surface of a sample cartridge on the prep station using the high sensitivity protocol pre-set. A microscope with CCD camera was used to capture images of the surface of the sample cartridge where the barcodes were bound and gene transcripts were counted. The barcodes were decoded and cross-referenced to a specific target resulting in actual counts of specific target molecules in a particular sample (Gene expression assay user manual, NanoString Technology, Seattle, WA). Zipped RCC files were received from NanoString Technology. NanoString RCC data files were imported into the nSolver 3 software (nSolver Analysis software, v3.0) where they were normalized to housekeeping genes. The housekeeping genes had an average read count four fold higher than the negative controls and any gene with counts less than 20 was reported to be negative. The analysis was done, in consultation with a biostatistician at the University of Queensland, using JMP statistical software (SAS Institute for comparing gene expression) and the nil and Ag/nil ratio of the gene expression between the TB, TB-THG and TB-T2D groups and between LTBI and LTBI-T2D were analysed.

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2.1.7 Enzyme linked immunosorbent assay (ELISA) in ALERT samples

Stored plasma samples were thawed on the day the ELISA was done and all the reagents were brought to RT before being used.

i. Elabscience® ELISA kit: Serotonin (5-hydroxytryptamine) ELISA

The serotonin competitive ELISA (Biocom, California, USA) was performed following the manufacturer's instructions. Briefly, 50 µl of the samples, standards and blank were added in duplicate to the pre-coated plate to the positions indicated on the template. Fifty µl of biotinylated detection antibody was added to the wells and the plates incubated for 45 minutes at 37 °C. After the incubation step, the plates were washed three times. Hundred µl Avidin-Horseradish Peroxidase (HRP) was added to each well and the plates incubated for 30 minutes. The plates were washed five times before 90 µl of Tetramethylbenzidine (TMB) substrate was added. The plates were again incubated for 15 minutes after which 50 µl sulfuric acid was added to stop the reaction. Colorimetric changes were detected at 450 nm using an Imark microplate reader (Bio-rad Laboratories, California, USA). A standard curve ranging from 15.63-1000 pg/mL was used and the Microplate manager 6 software, (Bio-rad Laboratories) used to calculate the serotonin concentrations in the samples.

ii. Abnova 3 kit Catecholamine ELISA: Adrenaline/Noradrenaline/Dopamine ELISA kit

The hormones in question were first extracted before the samples were analysed using the catecholamine ELISA kit (Abnova, Taipei City, Taiwan). Extractions were done according to the manufacturer's instruction and using the extraction plates provided. Briefly, 10 µl of the standards, controls and 300 µl of the plasma samples were added to the plates followed by 250 µl of deionized water. Assay and extraction buffer were then added to each well and the plates incubated for 30 minutes at RT while shaking at 600 rpm (all subsequent incubation steps were done at RT while shaking). Each well was washed twice and the plates incubated for five minutes between each wash step. Acylation buffer (150 µl) and acylation reagent (25 µl) were added to each well followed by a 15 minute incubation. After washing and drying the plate, hydrochloric acid (0.025 M; 175 µl) was added to all the wells and the plate incubated in the dark for 10 minutes. Thereafter, the samples were ready to be loaded onto the three different ELISA plates:

For the dopamine ELISA, 25 µl of the Catechol-o-methyltransferase enzyme solution was added to the microtiter strips of the dopamine ELISA plate followed by 50 µl of the isolated samples and 25 µl of the controls and standards, to allow for the formation of methyldopamine. Hydrochloric acid (0.025 M; 25 µl) was added to all the wells and the plate incubated for 30 minutes. Fifty µl rabbit anti-dopamine antibody was added and the plate incubated for two hours. The plates were washed three times, before 100 µl anti-rabbit immunoglobulin-

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peroxidase conjugate was added. Thereafter the plate was incubated for 30 minutes. After the 30 minutes incubation step, the plates were washed and 100 µl TMB substrate solution added. After 25 minutes, 100 µl of sulfuric acid was added to the plate to stop the reaction. The plates were read at 450 nm using an Imark microplate reader (Bio-rad Laboratories) and the concentrations calculated using the Microplate manager 6 software (Bio-rad Laboratories). A 4-parameter logistic regression standard curve was used and ranged from 57.4-15054 pg/mL. The measured concentrations were divided by 60 (as recommended by the manufacturer) to obtain the actual dopamine concentration.

For the noradrenaline ELISA, 25 µl of enzyme solution (Catechol-O-methyltransferase) was added in the noradrenaline microtiter strip. Twenty µl of the extracted standards, controls and samples were added to appropriate wells and incubated for 30 minutes. Fifty µl of rabbit anti-noradrenaline antibody was added in all wells and the plates incubated for two hours. Subsequent steps were done similarly as for dopamine. A standard curve ranging from 51-14251 pg/mL was used, and the measured concentration divided by 30 (as recommended by the manufacturer) to obtain the actual noradrenaline concentration.

Lastly, for the adrenaline ELISA, 25 µl of enzyme solution (Catechol-O-methyltransferase) was pipetted into the adrenaline microtiter strips. Hundred µl of the isolated samples, controls and standards were added to their respective wells followed by a 30 minute incubation. Fifty µl rabbit anti-adrenaline antibody was added in all wells and the plates incubated for two hours. Subsequent steps were performed as previously described for dopamine and noradrenaline. The standard curve with the lowest detection limit of 9.1 and highest detection limit of 426 pg/mL was used and the measured concentrations divided by 30 to obtain the actual concentration. All quality controls (QCs) were within the expected ranges.

iii. Elabscience® ELISA kit: Interferon inducible T cell alpha chemoattractant (I-TAC) sandwich ELISA

Hundred µl of neat serum samples, standards and blank were added to the pre-coated I-TAC ELISA plate (E-EL-H0051, Elabscience) and incubated for 90 minutes at 37 °C. The plates were washed three times. After the wash steps, 100 µl of Avidin-HRP conjugate was added to the wells and the plate incubated for 30 minutes. After washing the plates five times, 90 µl of substrate solution was added and again incubated for 15 minutes. Fifty µl of sulfuric acid was used to stop the reaction and the optical densities read at 450 nm using the Imark microplate reader (Bio-rad Laboratories). A standard curve ranging from 62.5-4000 pg/mL was used to calculate the unknown concentrations using the Microplate manager 6 software (Bio-rad Laboratories).

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iv. Elabscience® ELISA kit: Interleukin 22 (IL-22) ELISA in ALERT and TANDEM samples

Serum samples from both the ALERT and TANDEM study were included in the IL-22 ELISA (E-EL-H0106, Elabsciences). The ELISA plates were pre-coated with an Ab specific to human IL-22. Standards and neat samples were added to the plates in duplicate and the plates incubated for 90 minutes at 37 °C. One hundred µl of the biotinylated capture antibody was added after removing the samples and the plate incubated for one hour. The plates were washed three times with wash buffer and 100 µl of Avidin-HRP conjugate added to the wells. After a 30 minutes incubation step, the plates were washed five times and 100 µl of substrate reagent was added. The plate was again incubated for 15 minutes, after which the 50 µl sulfuric acid was added to stop the reaction. The colorimetric changes were immediately detected at 450 nm using the Imark microplate reader (Bio-rad Laboratories) and a 4 parameter logistic regression standard curve ranging from 15.63-1000 pg/mL was used. The Microplate manager 6 software (Bio-rad Laboratories) was used to calculate the IL-22 concentrations in the samples.

2.1.8 Luminex in ALERT samples

A customized multiplex screening assay from RnD Systems (Qiagen) was used to determine the concentration of 14 immune proteins namely; IFN-γ, IL-1β, IL-4, IL-6, IL-8, IL-10, IL-13, IL-18, IL-21, IL-22, IL-23, IL-27, IL-33, and TNF-α. Two single-plex assays were used to measure the concentrations of Oncostatin (OSM) and IL-35 both from Merck (Merck- Millipore).

i. Human Magnetic Luminex®: RnD Screening assays (14-plex)

Serum samples were diluted (1:2) and the assay done according to the manufacturer's instructions. Fifty µl of the micro particle cocktail was added into each well in the 96-well plate followed by 50 µl of the samples and standards. The plates were sealed and incubated for two hours at RT in the dark while shaking at 800 rpm. The plates were washed three times. Fifty µl of biotinylated antibody cocktail was added and the plates incubated for one hour, followed by three wash steps. The plates were incubated for 30 minutes after the addition of 50 µl of streptavidin-phycoerythrin (PE) conjugate and washed three times. Finally, wash buffer was added and the plates left on the shaker for two minutes to re-suspend the beads. The sample was read immediately on the Bio-Plex 200 instrument (Bio-rad Laboratories) and a four parameter logistic regression standard curve was used to determine concentrations of all the cytokines in the samples using the Bio-Plex Manager software, version 6 (Bio-rad Laboratories). The standard curve for each analyte was ranging from; IFN-γ 43.2-11220 pg/mL, IL-1β 15.8-3840 pg/mL, IL-13 454.8-110520 pg/mL, IL-33 47.8-11500 pg/mL, IL-8 4.4-1060 pg/mL, IL-10 3.3-790 pg/mL, IL-22 16.4-3970 pg/mL, TNF-α 8.9-2170 pg/mL, IL-21 29.4-

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7140 pg/mL, IL-23 228.8-55600 pg/mL, IL-6 4.8-1180 pg/mL, IL-18 54.6-13260 pg/mL, IL-27 375.7-91290 pg/mL and IL-4 15.4-3750 pg/mL.

ii. Milliplex® Map Kit: IL-35 and Oncostatin luminex

Luminex plates were primed with assay buffer for 10 minutes at RT while shaking at 600 rpm. The contents were discarded and 25 µl of standards and quality controls added to the wells as indicated on the plate template. Twenty five µl of assay buffer was added to all the sample wells and 25 µl of serum matrix to the wells containing the standards and quality controls. The diluted serum sample for IL-35 (1:4) and for OSM (1:2) were added to the sample wells containing assay buffer. Twenty five µl of beads were added to all the wells and the plates incubated for 16 hours, at 4 °C while shaking. After the incubation step, the plates were washed three times. Twenty five µl of detection antibody was added to each well and the plates incubated for one hour at RT (shaking). Following incubation, 25 µl of streptavidin-PE conjugate was added, without removing the contents of the wells, followed by a 30 minute incubation step. After washing the plate three times, 150 µl of sheath fluid was added and the plate left on the shaker for five minutes to re-suspend the beads. The mean fluorescent intensities were read on the Bio-Plex 200 instrument (Bio-rad Laboratories). The standard curve ranging from 6.9 to 5000 pg/mL for OSM and 0.8 to 800 ng/mL for IL-35 was used to calculate unknown cytokine concentrations using the Bio-Plex Manager software, version 6. The actual concentrations were obtained by multiplying by the dilution factor. All QCs were within the expected ranges.

2.1.9 Flow cytometry: TANDEM study

i. Thawing of peripheral PBMCs

Thawing media (TM), prepared by adding 10% of heat inactivated FBS (HyClone; BD) in RPMI (Biowest) with L-Glutamine (Sigma-Aldrich), was heated to 37 °C in a water bath prior to adding it to the cells. Cryopreserved PBMCs were thawed in a 37 °C water bath. One ml of the TM was added in a dropwise manner to the cells. The cells were gently mixed with the TM and transferred to a 15mL tube containing 8 mL of TM. The cells were centrifuged at 250 xg for 10 minutes, the media discarded and the cells again washed with 10 mL TM. After the second wash step, the cells were resuspended in 1 mL TM. PBMCs were counted, as described in section 2.1.3 iv, to determine the cell number and viability.

ii. Surface staining of PBMCs

PBMCs ($2.5x10^5$ cells) were centrifuged at 250 xg for five minutes and washed with 1 mL of PBS (All wash steps were followed by centrifugation). PBMCs were first stained with the Live/dead (Zombie aqua; BD) stain, diluted to 1:100 in PBS, and incubated for 15 minutes at 4°C. After the incubation step, the cells were first washed with 1 mL of PBS and secondly with

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1 mL of FACS buffer (2 % FBS in PBS). A MAIT cell antibody panel was used to stain the cells and included pre-titrated anti-human antibodies purchased from BD and BioLegend (Cambridge, UK). The panel include CD26-FITC (1:5 dilution, 10 µl; BD), anti-human CD8-APC-Cy7 (1:20 dilution, 2.5 µl; BD), anti-human CD4-BV412 (1:20 dilution, 2.5 µl; BD), anti-human CD3-Per-Cp (1:40 dilution, 1.25 µl; BD), anti-human TCR Vα7.2-APC (1:10 dilution, 5 µl; BioLegend), and anti-human CD161-PEcy7 (1:20 dilution, 2.5 µl; BioLegend) monoclonal antibodies. Cells were incubated with the antibody panel for an hour at 4 °C. The cells were washed after 30 minutes with FACS buffer. After washing with FACS buffer, PBMCs were fixed with 4 % formalin for 15 minutes in the dark. Formalin was washed off with 1 mL FACS buffer and the cells resuspended in 100 µl FACS buffer. Samples were kept at 4 °C until the samples were acquired on the FACSCanto II flow cytometry instrument with FACS Diva software, version 10 (BD). The results were analysed on FlowJo v10.1 software (TreeStar, Ashland, OR).

2.1.10 Statistical analysis

For the multiplexing data, values below and above the standard curve range, were extrapolated by the Bio-Plex Manager Software. Statistical analysis of clinical data was done using GraphPad PRISM version 6 (GraphPad Software Inc., California, USA). For more than two groups a Kruskal-Wallis analysis of variance (ANOVA) with a Dunn's *post hoc* test to correct for multiple comparisons was used. When testing for significant differences between two groups a non-parametric Mann-Whitney U test was used. Statistical analysis of MAIT cell frequencies, cytokine, hormone and CFU data was done using Statistica version 13 (StatSoft, Ohio, USA). The data were analysed using a repeated measures ANOVA with a Fisher LSD *post hoc* test. Correlations between groups were determined by Spearman correlations on GraphPad PRISM version 6 (GraphPad Software). Gene expression analysis was done using JMP statistical software version 13.2 (SAS institute, North Carolina, USA) and significance was assessed using repeated measures ANOVA and Dunns correction Ingenuity pathway analysis software (QIAGEN) was used to generate pathways for the differentially expressed genes.

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CHAPTER 3

Identification of distinct immune gene expression signatures in active TB patients and close contacts with and without type 2 diabetes

3.1. Introduction

Progression from LTBI to active TB disease occurs when the immune system fails to control the infection (Sutherland et al. 2014). Transcriptional analysis of whole blood and Mtb antigen stimulated blood has become an important tool to study the expression of immune system related genes and shows promise in identifying possible biomarkers for TB progression and treatment responses (Cliff et al. 2013; Zak et al. 2016; Thompson et al. 2017). Transcriptomic analysis of healthy controls, patients with T2D and TB patients with and without T2D showed there are shared elements with published TB signatures in TB patients without T2D (Prada-Medina et al. 2017; Berry et al. 2010). In the same study, increased neutrophil counts were associated with TB disease severity in patients with TB-T2D as previously reported in TB patients (Prada-Medina et al. 2017; Berry et al. 2010). Interestingly, pathways correlating to diabetic associated complications including several epigenetic reprogramming pathways were overexpressed in patients with TB-T2D, compared to patients with T2D only (Prada-Medina et al.2017). In this study, differentially expressed genes in TB-T2D patients included the four genes of the Maertzdorf TB diagnostic signature (Maertzdorf et al. 2015) as well as nine of the 16 genes of the Zak signature predicting TB progression within two years of infection (Zak et al. 2016). the fact that a gene expression profile exists which predicts which individuals who are LTBI positive will develop TB disease, our aim was to identify altered gene expression profiles in individuals with LTBI as well as TB patients with and without T2D and to describe the underlying molecular mechanisms that could contribute to the increased susceptibility to TB observed in these participants. In addition, we aimed to describe changes in gene expression profiles at the end of TB treatment. Pathway analysis was done in an attempt to understand alterations in immune responses in the different patient groups.

3.2 Methods

The detailed methods were discussed in chapter 2. Ex-vivo and Mtb antigen stimulated whole blood gene transcripts from CCs with LTBI with, and without T2D and TB patients with and without T2D (BL and M6) were measured using the NanoString Technology. CCs of TB patients with and without T2D were recruited as part of the ALERT study and TB patients with and without T2D were enrolled as part of the EU FP7 funded TANDEM study. Briefly, whole blood was added to the three tubes of the QFT assay, incubated overnight and the supernatant and cells harvested. The supernatants were stored at -80°C, and the pellet resuspended in RNA*Later,* which preserves RNA and then stored in -80°C. The pellet stored in RNA later was later thawed and total RNA extracted using Ribopure Ambion RNA isolation kit following the

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manufacturer's instructions. Total RNA was quantified using the Nanodrop as well as the RNA Qubit kit.

RNA of unstimulated (nil) and Mtb antigen stimulated (antigen tube) whole blood of TB patients (TB n=9, TB-THG n=5 and TB-T2D n=7; BL and M6) and individuals with LTBI (LTBI-noT2D n=20, LTBI-T2D n=20) were shipped to NanoString Technologies (in Seattle Washington) to determine the gene expression of 594 genes (579 immunological genes and 15 housekeeping genes) as part of the nCounter GX Human Immunology kit V2. Statistical significance was assessed using repeated measures ANOVA and Hochberg's correction between three groups and student T-test used to determine differences between two groups.

3.3 Results

3.3.1 Demographic and clinical characteristics

Participants with LTBI (n=20) and LTBI-T2D (n=20) and 21 TB patients (TB n=9, TB-THG n=5 and TB-T2D n=7) were included in this study. No significant differences were seen in age between the groups (Table 3.1). The clinical parameters for the LTBI and LTBI-T2D groups were taken at a single time point and for TB, TB-THG and TB-T2D at BL and M6. At BL, the median BMI in LTBI and LTBI-T2D was significantly higher compared to TB and TB-THG. The median BMI at M6 was significantly higher in the TB-T2D group compared to both the TB and TB-THG groups, as shown in (Table 3.1). The median HbA1c was significantly higher in the LTBI-T2D and TB-T2D groups compared to LTBI and TB (Table 3.1), respectively. At BL, there were differences in HbA1c between TB-THG and TB-T2D although not significant, however, significant differences were observed in TB-THG and TB-T2D at M6 (Table 3.1). Even though the HbA1c is much higher in the TB-T2D group it did not reach significance due to the low power because of the small sample size. We measured High density lipoprotein (HDL), Low density lipoprotein (LDL), triglycerides and total cholesterol (CHL) in LTBI and LTBI-T2D at BL. Triglycerides were significantly higher in LTBI-T2D (vs. LTBI), but no significant differences were observed in HDL, LDL and total CHL. In TB-THG and TB-T2D, the lipid measurements were performed at week 2 and at M6 and no significant differences were observed. Generally, the lipid concentrations were elevated in individuals with T2D irrespective of LTBI or TB status, with the exception of HDL, which was lower. With respect to medication, 50 % of the LTBI-T2D patients were on metformin, 25 % on insulin and 80 % were on a combination of medication and none of them were on prophylactic LTBI treatment in line with TB treatment policies in South Africa. All TB-T2D patients were on TB standard treatment, 100 % were on metformin, 71% on insulin and 57% taking a combination of medication. The demographic and clinical information is summarised in Table 3.1.

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Table 3.1 Demographic and clinical parameters of LTBI, LTBI-T2D, TB, TB-THG and TB-T2D patients

Group	LTBI (n=20)	LTBI-T2D (n=20)	TB (n=9)	TB-THG(n=5)	TB-T2D(n=7)	P-value
T2D history and management						
T2D medication						
Metformin (%)	-	10(50)	-	-	7(100)	
Insulin (%)	-	5(25)	-	-	5(71)	
Other medication (%)	-	16	-	-	4	
Known diabetic status	-	11	-	-	5	
Sociodemographic						
Age, median (IQR)	46.5(12.0)[a]	56(12.3)[a]	48(5.5)[a]	48(10.5)[a]	46(7.0)[a]	0.0827
Sex, number (%)						
Female	13(65)	17(85)	4(44)	2(40)	5(71)	
Male	7(35)	3(15)	5(56)	3(60)	2(29)	
BMI, median (IQR)						
Baseline	25.0(10.2)[b]	28.5(6.5)[b]	18.4(5.7)[a]	18.2(0.7)[ac]	26.0(6.9)[abc]	**<0.0001**
Month 6	-	-	19.1(5.1)[a]	19.1(2.4)[a]	26.6(7.6)[b]	**0.0055**
Clinical information, median (IQR)						
RBG (mmol/L)						
Baseline	-	-	5.7(1.9)[a]	6.0(3.7)[a]	[#]18.35(4.1)[a]	0.0513
FBG(mmol/L)	4.8(1.6)[a]	9.1(7.9)[b]	-	-	-	**<0.0001**
HbA1c (%)						
Baseline	5.7(0.78)[a]	7.9(3.8)[b]	5.7(0.5)[a]	6.7(0.4)[ab]	12.0(1.9)[b]	**<0.0001**
Month 6	-	-	5.5(0.7)[a]	5.7(0.6)[a]	10.7(5.3)[b]	**0.0014**
Creatinine (µmol/L)						
Week2	-	-	-	53.5(40.5)[a]	56.0(19.0)[a]	0.4678
Month 6	-	-	-	[#]49.5(61.0)[a]	51.0(3.0)[a]	0.4762
Total CHL (mmol/L)						
BL	4.6(1.1)[a]	5.1(2.2)[a]	-		-	0.1403
Week2	-	-	-	-	4.8(2.8)[a]	0.7715

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Month 6	-	-	-	4.5(1.7)[a]	5.2(3.1)[a]	0.0952
HDL (mmol/L)				[#]4.0(0.3)[a]		
BL	1.4(0.4)[a]	1.2(0.5)[a]	-		-	0.2229
Week2	-	-	-	-	1.0(0.5)[a]	0.3429
Month 6	-	-	-	1.4(0.6)[a]	1.2(1.3)[a]	0.5714
LDL (mmol/L)				1.5(1.3)[a]		
BL	2.5(1.1)[a]	3.1(1.4)[a]	-		-	0,1560
Week2	-	-	-	-	3.2(1.2)[a]	0.6857
Month 6	-	-	-	2.65(2.5)[a]	2.8(2.5)[a]	0.1333
Triglycerides (mmol/L)				[#]2.0(0.4)[a]		
BL	0.9(0.7)[a]	1.6(1.0)[b]	-		-	**0.0025**
Week2	-	-	-	-	1.3(0.6)[a]	0.1913
Month6	-	-	-	0.8(0.6)[a]	2.7(2.8)[a]	0.0952
				[#]1.1(0.5)[a]		

Data expressed as n (column %) unless specified. BMI-Body mass Index, RBG-Random blood glucose, Bold number indicates a significant ANOVA, P-value of <0.05 was considered significant using Kruskal-Wallis and Dunns post-hoc test. Letters indicate statistical significance if different from each other. Values with the same letter are not significantly different from each other. #- 2 participants in that particular group

3.3.2 Overall transcript expression in unstimulated and stimulated blood from LTBI, LTBI-T2D, TB, TB-THG and TB-T2D at BL

Gene expression analysis of a total of 594 immune genes was performed using the NanoString Technology platform to identify differentially expressed transcripts. The differential transcripts were investigated in the unstimulated and stimulated blood from individuals with LTBI with and without T2D and also in the TB, TB-THG and TB-T2D patients. The individuals with LTBI with and without T2D were compared to each other and TB, TB-THG and TB-T2D patients compared to one another. Gene transcripts data were reported as unstimulated, from the nil tube and stimulated, from the antigen tube divided by the value from the nil tube.

Figure 3.1A shows the number of total transcripts that were differentially expressed in TB compared to TB-THG in both stimulated and unstimulated samples, shown as stim TB-THG and unstim TB-THG, respectively. Figure 3.1A also shows total differentially expressed transcripts inTB compared to TB-T2D in both stimulated and unstimulated samples, shown as stim TB-T2D and unstim TB-T2D, respectively. Two hundred and fifty genes were upregulated and 329 downregulated in the unstimulated blood of individuals with LTBI compared to LTBI-T2D (Figure 3.1C). In the stimulated blood, 263 genes were upregulated and 315 downregulated in individuals with LTBI compared to LTBI-T2D (Figure 3.1C). Observed

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differences in the transcripts from unstimulated blood in TB patients compared to TB-THG and TB-T2D, shows that *ex-vivo* differences exist in the RNA of the investigated patients. Furthermore, the differentially expressed genes are shown on Venn diagrams to highlight the overlap of any of the differentially expressed transcripts between TB-THG and TB-T2D compared to TB. We found 182 and 187 of stimulated gene transcripts to be similarly downregulated and upregulated in TB-THG and TB-T2D compared to TB, respectively. Interestingly, 86 of the gene transcripts from stimulated whole blood were upregulated in TB-THG and downregulated in TB-T2D, in addition, 123 were downregulated in TB-THG and up regulated in TB-T2D (Figure 3.1B). The Venn diagram was also done for LTBI and LTBI-T2D to determine the unique and shared genes between them (Figure 3.1D). A total of 211 gene transcripts which were upregulated in unstimulated blood of LTBI-T2D individuals were downregulated in stimulated blood from LTBI-T2D compared to LTBI. Two hundred and twenty four gene transcripts were downregulated in unstimulated blood and upregulated in stimulated blood from LTBI-T2D. Furthermore, 104 gene transcripts downregulated in unstimulated blood from T2D patients were also downregulated in the stimulated blood, and 39 genes were similarly upregulated in unstimulated and also in RNA from stimulated blood of LTBI-T2D (Figure 3.1D). There is a contrast between TB compared to both groups with high glucose (TB-THG and TB-T2D) leading to upregulation of more genes (than downregulation), while individuals with LTBI compared to LTBI with high glucose shows more downregulated than upregulated genes (Figure 1A and D).

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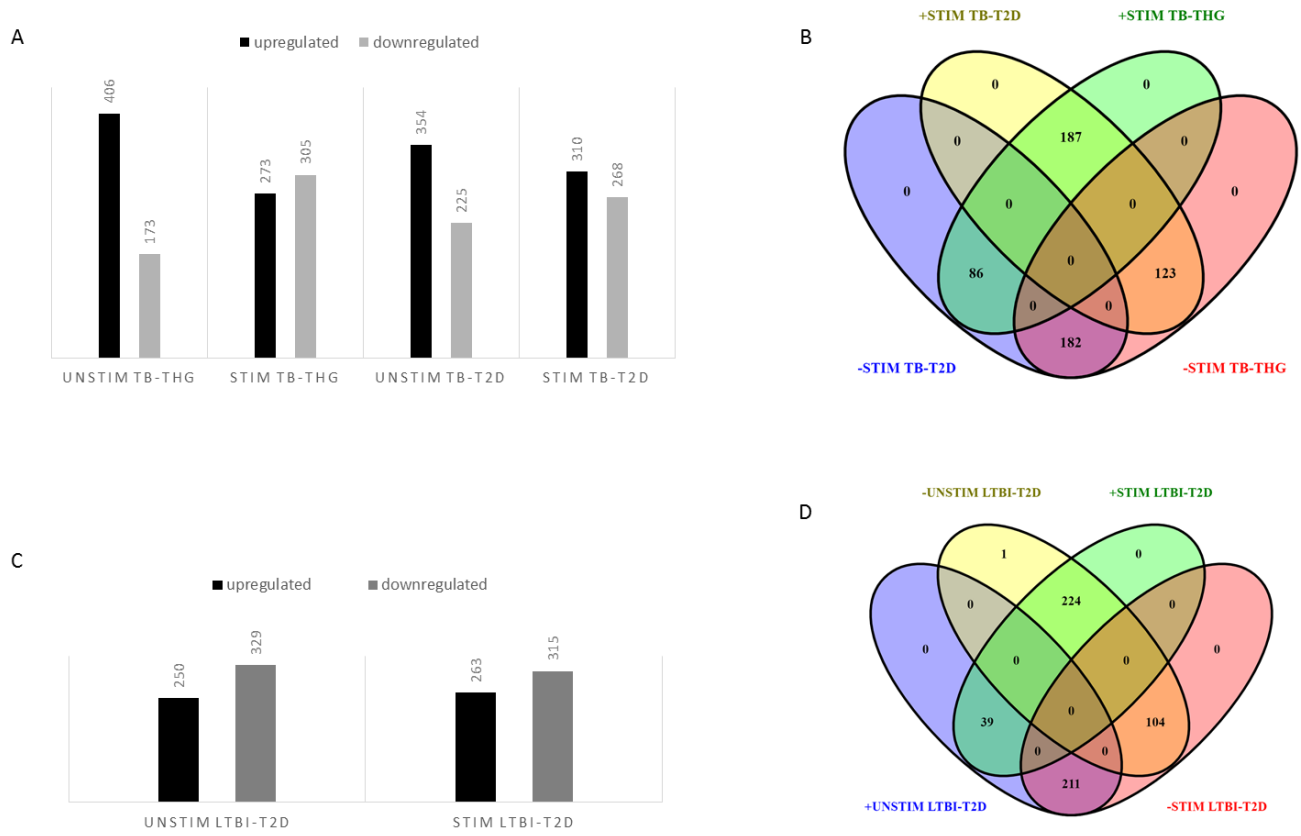

Figure 3.1 Overall transcriptomic gene expression in TB, TB-THG and TB-T2D. RNA was isolated from whole blood stimulated with Mtb specific antigen using QFT assay (nil, unstimulated and Ag, stimulated). Gene expression analysis of 594 genes as part of the nCounter GX Human Immunology kit V2 was done on the extracted RNA. Column graphs were generated on excel and Venny 2.0 software was used to generate Venn diagrams. Column graph showing the number of all upregulated and downregulated transcripts from RNA isolated from unstimulated and stimulated blood of TB patients compared to TB-T2D and TB-THG (A). A Venn diagram showing the number of all differentially expressed transcripts and the transcripts that are shared between the groups (B). Column and Venn diagrams showing the number of all upregulated and downregulated transcripts from RNA isolated from unstimulated and stimulated blood of LTBI individuals compared to LTBI-T2D (C and D).+ means upregulated and - means downregulated, unstim is forunstimulated, stim is for stimulated.

3.3.3 Differentially expressed blood transcripts in unstimulated whole blood of TB-THG and TB-T2D (vsTB) at baseline

To determine differentially expressed whole blood gene transcripts from TB, TB-THG and TB-T2D patients, heatmaps were used to determine the clustering of the gene expression data in the different groups. Gene expression was considered significantly different when the p value was < 0.01 and FDR-value was < 0.05 when compared to another group. Sixty-three genes were significantly different between TB-T2D and TB, of which, 25 were upregulated and 38 downregulated in the TB-T2D group (Figure 3.2A and C). Twenty-nine genes were differentially expressed between TB-T2D and TB-THG, of which, 25 were downregulated and four upregulated in the TB-T2D patients (Figure 3.2B and D). TB compared to TB-THG showed significant differences in the five genes (P selectin glycoprotein ligand 1 (SELPLG), membrane metallo-endonuclease (MME), intelectin 1 (ITLN1), complement component 1Q

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subcomponent binding protein (C1QBP) and B cell lymphoma like protein 11 (BCL2L11), (data not shown). Two genes were upregulated in TB-THG vs TB (SELPLG and MME) and three downregulated genes (ITLN1, C1QBP and BCL2L11). We used the Ingenuity pathway analysis (IPA) software to analyse differentially expressed genes from unstimulated blood between the TB and TB-THG, and found that pathways associated with innate immune responses including the complement system and apoptosis pathway were highly represented.

All significantly downregulated and upregulated genes from unstimulated blood at BL were analysed using Venn diagrams to enable visualization of shared and unique gene transcripts (Figure 3.2E, F). Twenty-two genes were exclusively downregulated in the TB-T2D (vs TB), two were significantly downregulated in TB-THG (vs TB) and 10 were specific to TB-T2D (vs TB-THG). BCL2L11, an apoptotic marker, was similarly downregulated in TB-THG and TB-T2D compared to TB. Fifteen gene transcripts (CCL2, CCL4, CCL3, IL-1A, cluster of differentiation 163 (CD163), CXCL2, plasminogen activator urokinase (PLAU), CXCL1, IL-1R1, tumour necrosis factor receptor superfamily member 8 (TNFRSF8), CCRL2, NF-KB1, proteasome non-ATPase regulatory subunit 7 (PSMD7), proteasome subunit beta type 5(PSMB5) and B cell lymphoma 2 associated X protein (BAX)) were downregulated when TB-T2D was compared to both TB and TB-THG (Figure 3.2E). The 15 gene transcripts may be of significance particularly, since these genes were downregulated in TB-T2D vs. TB and also found downregulated in TB-T2D vs. TB-THG. The genes that are altered by higher glucose appear to be in part similar between TB-T2D and TB-THG, but not ignoring the fact that differences between both groups exists which are not driven by excessively high blood glucose as shown by the distinct 15 gene signature between TB-T2D vs TB and TB-T2D vs TB-THG. These result shows the uniqueness of the 15 gene transcript profile to TB-T2D and may have impact in the pathogenesis and clinical outcomes associated with T2D during TB-T2D comorbidity. Twenty-four genes were exclusively upregulated in TB-T2D (vs TB), three were upregulated in TB-T2D (vs TB-THG) and two were specific to TB-THG (vs TB). One gene transcript (mitogen activated protein kinase activation protein kinase 2 (MAPKAPK2) was similarly upregulated in TB-T2D and TB-THG compared to TB (Figure 3.2F). Fewer gene transcripts were differentially expressed after stimulation, between patinets with TB-T2D compared to patients with TB. Results demonstrating changes in stimulated whole blood at BL are shown in supplementary Figure 1 A-C and month 6 gene expression shown in supplementary Figure 2A-C.

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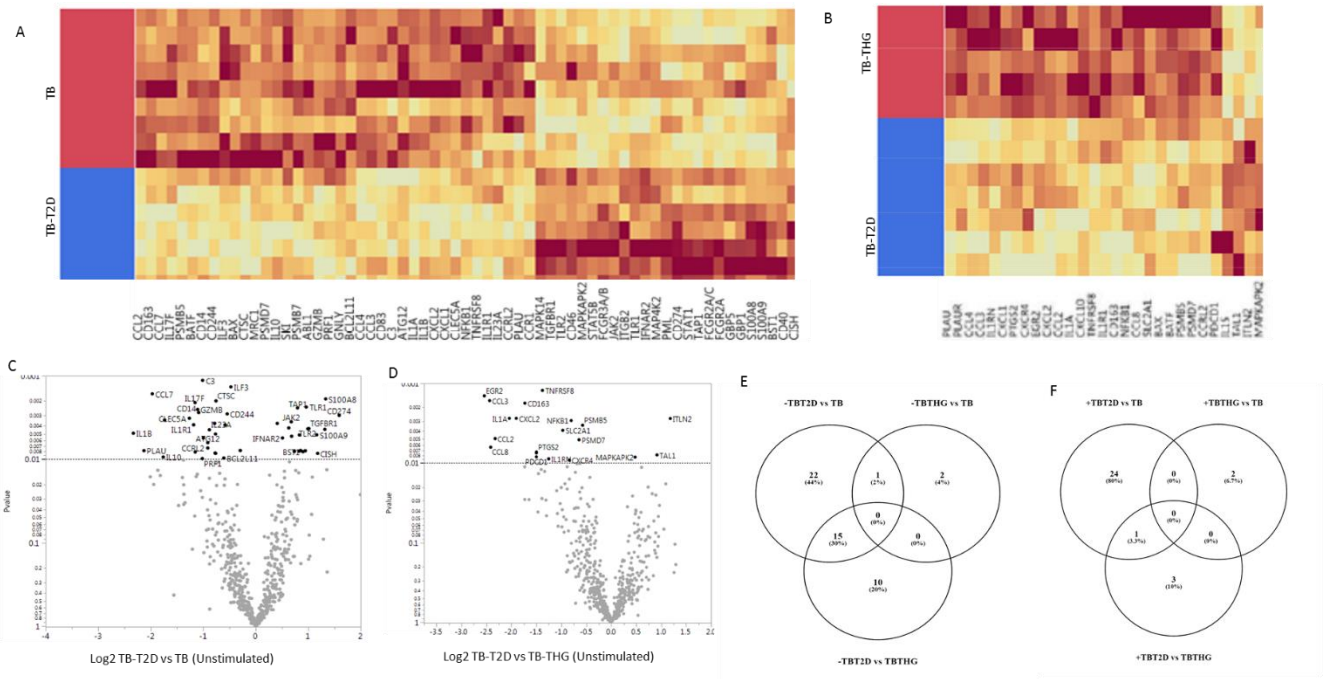

Figure 3.2: Differentially expressed gene transcripts between TB, TB-THG and TB-T2D. RNA was isolated from whole blood of TB-T2D, TB-THG and TB stimulated with Mtb specific antigen using QFT assay (nil, unstimulated only represented in the figure). Gene expression of 594 genes as part of the nCounter GX Human Immunology kit V2 was done on the extracted RNA. Down and upregulated transcripts from unstimulated blood of TB compared to TB-T2D (n= 14, A and C), and TB-THG vs. TB-T2D (n=12, B and D). Downregulated and upregulated genes commonly and exclusively expressed between the TB-T2D vs TB, TB-THG vs TB and TBT2D vs TBTHG (E and F). JMP SAS was used to generate heatmaps and volcano plots, Venny 2.0 software was used to generate Venn diagrams and used Student T test to determine statistical difference between the groups. Genes with a p value was < 0.01 and FDR < 0.05 was statistically significant. - downregulated, + upregulated. Figure A Red TB-THG, Blue TB-T2D, Figure B red TB, blue TB-T2D.

3.3.4 Differentially expressed gene transcripts in blood from LTBI with and without T2D

Analysis of differentially expressed genes was done to identify distinct genes associated with LTBI-T2D. Genes have been described showing differences between LTBI and TB, however, there is no published data on LTBI-T2D gene expression. There were 26 genes, 16 significantly upregulated and 10 significantly downregulated in unstimulated blood from LTBI-T2D compared to LTBI (Figure 3.3A). Stimulating with Mtb antigens increased the transcriptomic differences that we saw since stimulated blood resulted in 42 genes significantly expressed, and 36 of those were downregulated in LTBI-T2D patients compared to LTBI (Figure 3.3B).. There were seven genes exclusively upregulated in the unstimulated blood of LTBI-T2D patients (Figure 3.3E i) and nine that were exclusively downregulated in the LTBI-T2D patients (Figure 3.3E ii) compared to LTBI. After stimulation, there were five genes uniquely upregulated (Figure 3.3E iii) and 27 downregulated (Figure 3.3E iv) in the individuals with LTBI-T2D (vs LTBI). Interleukin-18 plays a critical role in immune response against Mtb infection (Schneider et al. 2010). Our results showed the IL-18 gene transcript to be the only

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one that was downregulated in the unstimulated and upregulated in the stimulated blood of LTBI-T2D (vs LTBI). Nine of the gene transcripts were upregulated in the unstimulated and downregulated in stimulated blood of individuals with LTBI-T2D (vs LTBI) (Figure 3.3E vi).

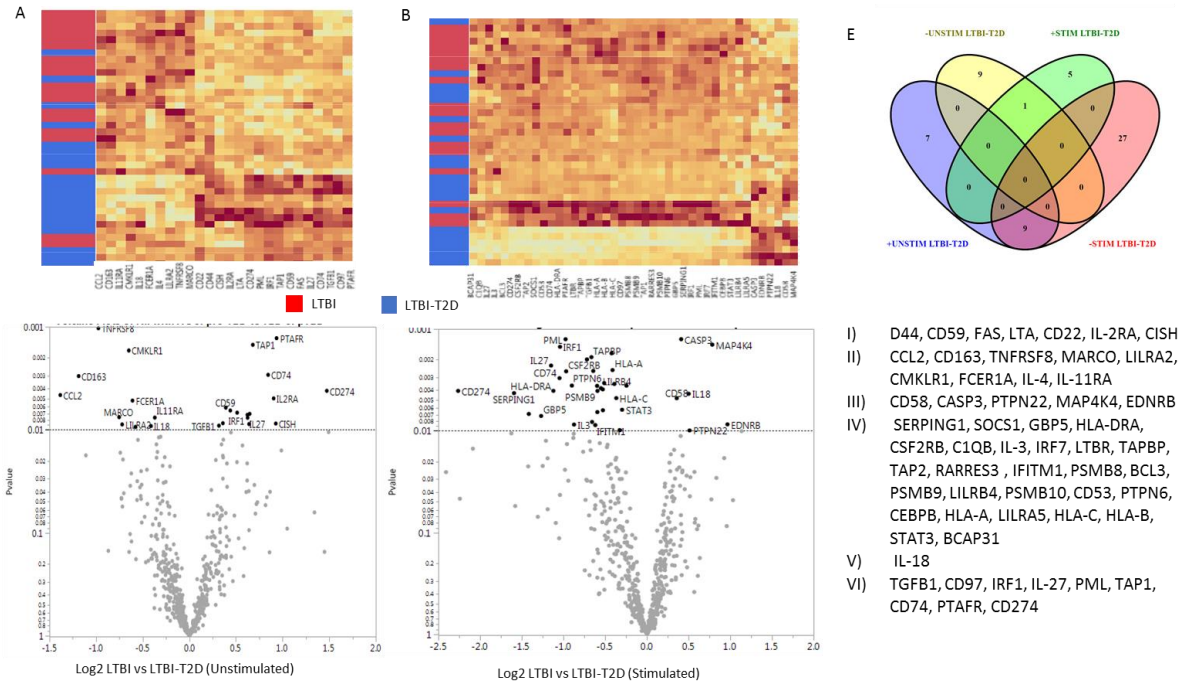

Figure 3.3: Differentially expressed gene transcripts in LTBI and LTBI-T2D. RNA was isolated from whole blood of LTBI-T2D and LTBI stimulated with Mtb specific antigen using QFT assay (nil, unstimulated and Ag, stimulated). Gene expression of 594 genes as part of the nCounter GX Human Immunology kit V2 was done on the extracted RNA. Significantly down and upregulated transcripts from unstimulated blood of LTBI-T2D compared to LTBI (A and C). Downregulated transcripts from stimulated blood of LTBI-T2D and LTBI (B and D). Downregulated and upregulated genes commonly and exclusively expressed between the LTBI-T2D vs LTBI are shown in 3E: I-VI. JMP SAS was used to generate heatmaps and volcano plots, Venny 2.0 software was used to generate Venn diagrams and used Student T test to determine statistical difference between the groups. Genes with a p value < 0.01 and FDR < 0.05 was statistically significant. – means downregulated, + means upregulated, unstim is for unstimulated, stim for stimulated, Red LTBI, Blue LTBI-T2D.

3.3.5 A core gene signature in whole blood differentiates TB-THG from TB-T2D patients at baseline

Differentially expressed genes were used to run a recursive partitioning analysis (RPA) to identify gene transcripts that can categorize patients in a group that describes their condition, either as TB, TB-THG or TB-T2D. The partition is created in such a way that gene expression with similar response values are grouped. After the partition is completed, a constant value of the response variable is predicted within each patient group (Sun et al. 2011). Figure 3.4A is a representative cubic cluster of patients depending on the expression levels of the following genes; PSMB5, complement component receptor 1 (C1R) and C1QBP from unstimulated blood at BL. PSMB5 with a log2 expression value of less than 6.1 predicted all TB-T2D patients with an area under the curve (AUC) of 1.00 (Figure 3.4B). To further characterize TB and TB-THG, C1QBP with a log2 expression value of < 6.7 was able to predict all five TB-THG patients (AUC=0.98; Figure 3.4B), but included one of the TB patients in this group. Subsequently, the

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addition of C1R, with a log2 expression value of < 0.2, could separate all TB from TB-THG patients with an AUC of 0.99 (Figure 3.4B). The gene signature consisting of PSMB5, C1QBP and C1R were therefore able to accurately discriminate the three patient groups. A univariate analysis was done on the three genes to describe their expression in the different test samples as shown in Figure 3.4C to F. PSMB5 was significantly lower in TB-T2D compared to both TB and TB-THG (p=0.0001). C1QBP was significantly higher in TB compared to both TB-THG and TB-T2D (p=0.0069) (Figure 3.4C-D). C1R was not different among the groups (p=0.7328; data not shown).

Furthermore, the RPA was done for the stimulated blood at BL and three genes differentiating TB-THG from TB-T2D were identified, LILRB4, LGALS3 and ABCB1. When the level of expression of LILRB4 was ≥ 1.02, the genes were able to identify all TB-THG, however at an expression level of ≤ 1.02 the gene was not able to discriminate between TB and TB-T2D. To further characterize TB and TB-T2D patients, the addition of galectin 3 (LGALS3) with the expression value of > 0.08 was able to identify all TB-T2D patients but included one TB patient in this group. Subsequently, the addition of ATP binding cassete subfamily B member 1 (ABCB1) with a log2 expression > -0.39 could separate all TB from TB-T2D patients with an AUC of 0.99. Using this approach we found a transcript signature in Mtb-antigen stimulated whole blood that can differentiates patients with TB-THG from TB-T2D (LILRB4, LGALS3 and ABCB1). A univariate analysis on these transcripts showed that leukocyte immunoglobulin like receptor B4 (LILRB4) was significantly higher in TB-THG compared to both TB and TB-T2D (p=0.0003) and that LGALS3 was significantly higher in TB-T2D compared to both TB and TB-THG (p<0.0001) (Figure 3.4E-F). ABCB1 was not different between the groups (p=0.5601) (data not shown).

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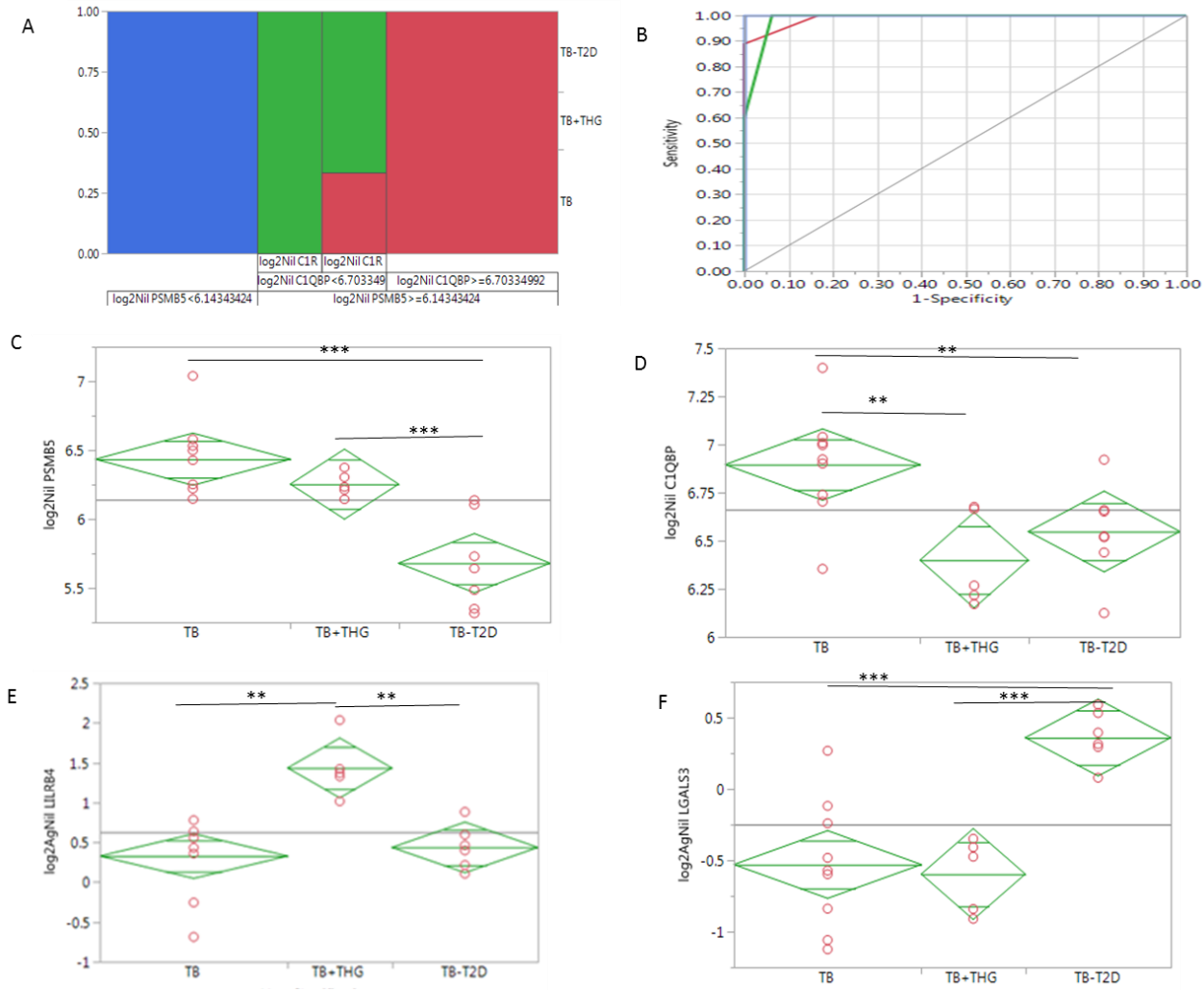

Figure 3.4: Gene signature distinguish between TB-THG and TB-T2D. (A) Cubic clustering from recursive partition analysis (RPA) of gene transcripts from RNA isolated from unstimulated blood of patients at baseline showing three markers accurately categorizing patients in either TB (red), TB-THG (green) and TB-T2D (blue). (B) Receiver operating characteristics curve showing the accuracy of the three markers to predict any of the patients as either TB, TB-THG or TB-T2D patients. Diamond plots showing differences in the expression of PSMB5, C1QBP in unstimulated blood (C and D) and LILRB4 and LGALS3 transcripts in stimulated blood (E and F). Mean expression was reported and ANOVA used to determine statistical significance. *p<0.01, **p<0.001, ***p<0.0001.

3.3.6 Gene signatures in whole blood could be associated with dysregulated pathways in LTBI-T2D that may increase susceptibility to TB

A group of differently expressed genes from unstimulated whole blood were used in RPA to identify a gene signature that would accurately classify LTBI with (n=20) and without T2D (n=20). Three genes cluster of differentiation (CD59), tumour necrosis factor ligand superfamily member 11 (TNFSF11) and cluster of differentiation (CD247) were able to distinguish between LTBI and LTBI-T2D. CD59 with a log2 expression value of ≥ 8.25 could identify 12 of the 20 patients as T2D. TNFSF11 (at a cut off < 3.85) and CD247 (at cutoff < 9.52) were added to the model to further distinguish the eight remaining T2D patients and by doing so, a three gene signature, could discriminate all LTBI from LTBI-T2D with an AUC of 1.00 (Figure 3.5A and B). In addition, significant gene transcripts from stimulated blood were

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also used for the RPA modelling. TNFRSF8, LILRA6 and PSMB7 were identified as good predictors for either LTBI or LTBI-T2D individuals. TNFRSF8 at a log2 expression value of ≥ 0.85, LILRA6 with a log2 value of ≤ 0.06 and PSMB7 with a log2 value of ≤ 0.12 were able to accurately classify patients as LTBI or LTBI-T2D with an AUC of 1.00 (data not shown). Most importantly, these gene signatures could be involved in immunological pathways that may render patients with T2D susceptible to TB.

A univariate analysis of the RPA predictive genes was done for both signatures from unstimulated and stimulated blood. CD59 was significantly higher in the LTBI-T2D compared to LTBI (p=0.0059) (Figure 3.5C). TNFSF11 (p=0.1573) and CD247 (p=0.7727) were not significantly different between the two groups in unstimulated blood (data not shown). From the stimulated samples, TNFRSF8 was significantly higher in the LTBI-T2D compared to LTBI (p=0.0097) (Figure 3.5D). Leukocyte immunoglobulin like receptor A6 (LILRA6) (p=0.3137) and PSMB7 (p=0.6046) were not significantly different (data not shown).

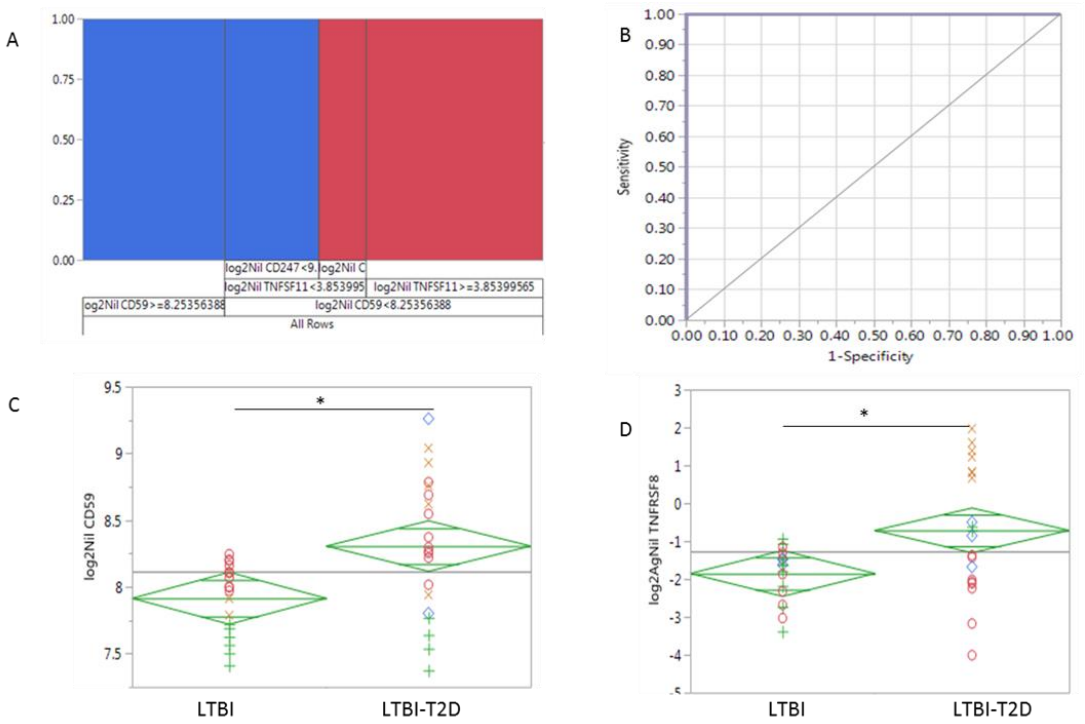

Figure 3.5: Gene signature distinguishing between LTBI and LTBI-T2D. (A) Cubic clustering from recursive partition analysis (RPA) of gene transcripts from RNA isolated from unstimulated blood of study participants highlighting three markers that shows distinct immune signature in LTBI-T2D (blue) compared to LTBI (red) (B) Receiver operating characteristics curve of unstimulated blood at baseline showing the accuracy of the three markers to identify altered immune genes in LTBI-T2D compared LTBI. Diamond plots showing differences in the expression of CD59 and TNFRSF8 transcripts in LTBI-T2D compared to LTBI (C and D). *p<0.01, **p<0.001, ***p<0.0001.

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3.3.7 The number of differentially expressed gene transcripts was reduced in TB-T2D patients at month 6

The transcript expression profile at M6 was investigated to determine how TB treatment would affect immune gene expression in the different patient groups. When comparing the gene expression between BL and M6, seven genes were differentially expressed in TB-THG (compared to TB). Platelet and endothelial cell adhesion molecule 1 (PECAM1), integrin subunit alpha 6 (ITGA6), CCR7, membrane spanning 4 domains A1 (MS4A1) and transcription factor 7 (TCF7) were significantly upregulated, while toll interleukin 1 receptor domain containing adapter molecule 1 (TICAM1) and IL17A were downregulated (data not shown). At M6, however, none of the genes were differentially expressed between TB-THG and TB (Figure 3.6A). In TB-T2D (vs TB) we observed a drastic reduction in differentially expressed genes at M6 compared to BL. There were now eight genes downregulated (TICAM1, PSMD7, chromosome 14 open reading frame 166 (C14orf166), BCL2L11, PSMB7, mothers against decapentaplegic homolog 5 (SMAD5), Interleukin enhancer binding factor 3 (ILF3) and CXCL10) and two genes upregulated (PECAM1 and B cell linker protein (BLNK)) in TB-T2D (vs TB) at M6 (Figure 3.6B). Even after the decrease in the total number of differentially expressed genes in TB-T2D from BL to M6, there were four genes (ILF3, PSMD7, PSMB7 and BCL2L11) which were downregulated at both time points. At M6, five genes (TP53, SMAD5, PSMC2, C14orf166 and C1QBP) were downregulated in TB-T2D compared to TB-THG (Figure 3.6C). PECAM1 was upregulated and TICAM1 downregulated in TB-T2D compared to TB at M6, the same was seen when comparing TB-THG to TB at M6. At the end of treatment, the reduction in gene transcripts and loss of differential expression is expected as the patients are cured. It is possible that the immune response is returning to normal, while the differences that remain are specific to T2D.

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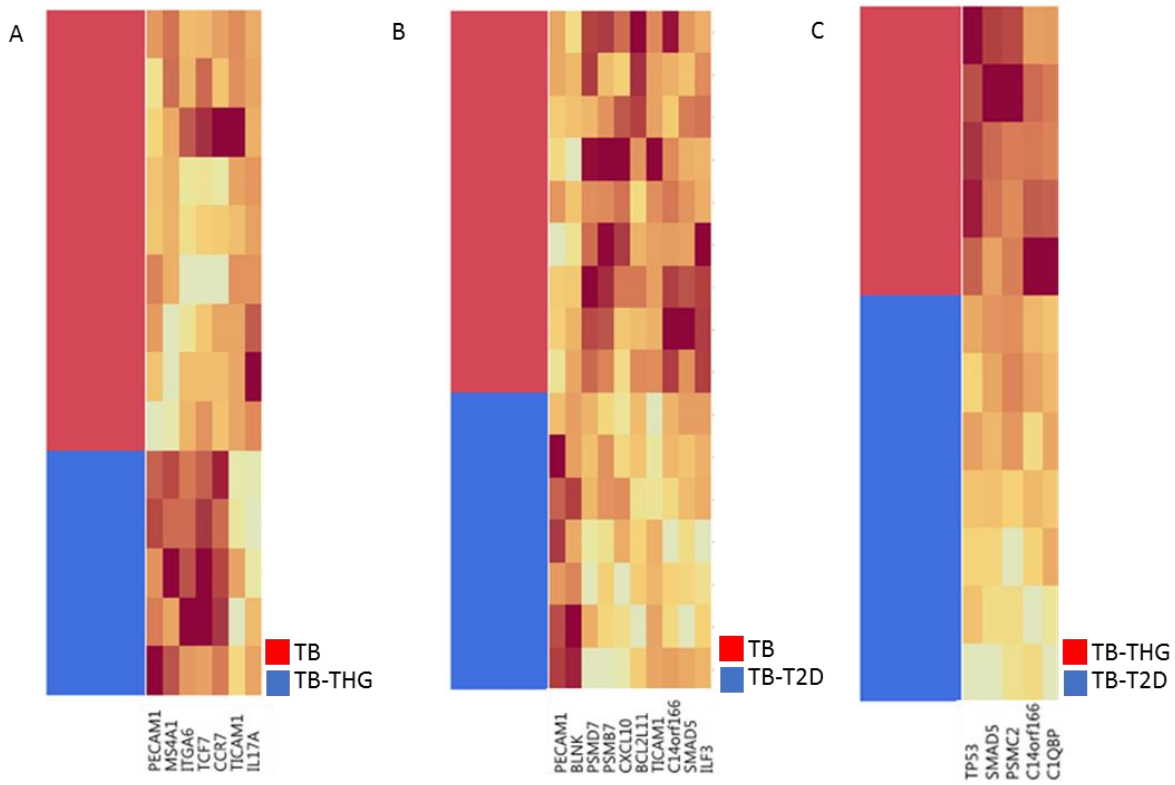

Figure 3.6: Differentially expressed gene transcripts between TB, TB-THG and TB-T2D at month 6. RNA was isolated from whole blood of TB-T2D, TB-THG and TB stimulated with Mtb specific antigen using QFT assay (nil, unstimulated only represented in the figure). Gene expression of 594 genes as part of the nCounter GX Human Immunology kit V2 was done on the extracted RNA. Down and upregulated transcripts from unstimulated blood of TB compared to TB-THG (A), TB vs. TB-T2D (B) and TB-THG vs TB-T2D. JMP SAS was used to generate heatmaps and used Student T test to determine statistical difference between the groups. Genes with a p value < 0.01 and FDR < 0.05 was statistically significant. Figure A Red TB, Blue TB-THG, Figure B red TB, blue TB-T2D and Figure C red TB-THG and blue TB-T2D.

3.3.8 Antigen presentation and Th1 activation and glucocorticoid signalling pathways are dysregulated in whole blood from LTBI, LTBI-T2D, TB, TB-THG and TB-T2D participants

To investigate the role of the genes that were differentially expressed in the study groups, we performed an IPA analysis. Genes from unstimulated and stimulated whole blood, which were significantly different between LTBI-T2D and LTBI revealed alterations in immune responses such as antigen presentation and Th1 activation pathway (Table 3.2). From the stimulated whole blood of TB compared to TB-T2D at BL, the differentially expressed genes were involved in the regulation of triggering receptor expressed on myeloid cells (TREM-1) signalling which is critical for innate immune responses including activation of inflammatory responses (Colonna and Facchetti, 2003). TREM-1 is poorly expressed in Mtb infection, and correlates with inflammatory response rather than with bacterial proliferation (Colonna and Facchetti, 2003). In our results, TREM-1 could be induced by T2D in TB patients. Glucocorticoid receptor signalling was also among the overrepresented pathways in TB-T2D patients compared to TB. This could be associated with the IL-10 production that is seen in TB-T2D patients, because glucocorticoids suppress pro-inflammatory responses, which may

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enhance the secretion of other anti-inflammatory molecules (Schaaf and Cidlowski, 2003). The complement and apoptosis pathways were observed when genes differentially expressed between TB and TB-THG from unstimulated whole blood at BL were investigated. After stimulating whole blood with Mtb-antigens, pathways involving IL-22 signalling, IL-15 production and type 1 interferon signalling were overrepresented in TB-THG at BL (Table 3.2). The identified pathways are critical for immune response during TB and their differential expression shows that there are major differences between patients with and without T2D, and need to be further investigated to understand the biological functions in the individual groups.

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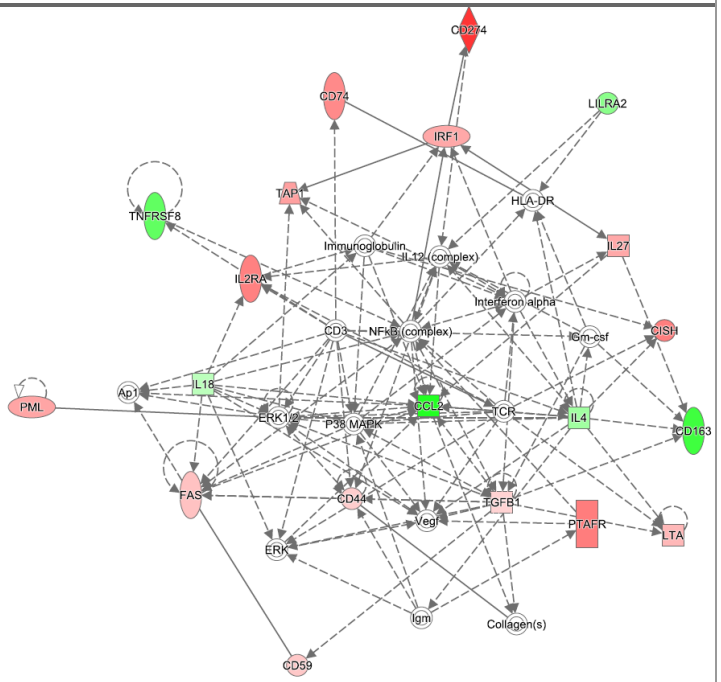
Table 3.2: IPA of differentially regulated genes in the study participants

	canonical pathways	Diseases	Molecular function	Physiological systems development	Network
Unstimulated LTBI vs LTBI-T2D	Th1 and Th2 Activation Pathway Th1 Pathway Altered T Cell and B Cell Signalling in Rheumatoid Arthritis Role of Cytokines in Mediating Communication between Immune Cells HMGB1 Signalling	Infectious Diseases Dermatological Diseases and Conditions Organismal Injury and Abnormalities Inflammatory Response Immunological Disease	Cell Death and Survival Cell-To-Cell Signalling and Interaction Cellular Development Cellular Growth and Proliferation Cellular Compromise	Connective Tissue Development and Function Tissue Development Haematological System Development and Function Haematopoiesis Lymphoid Tissue Structure and Development	

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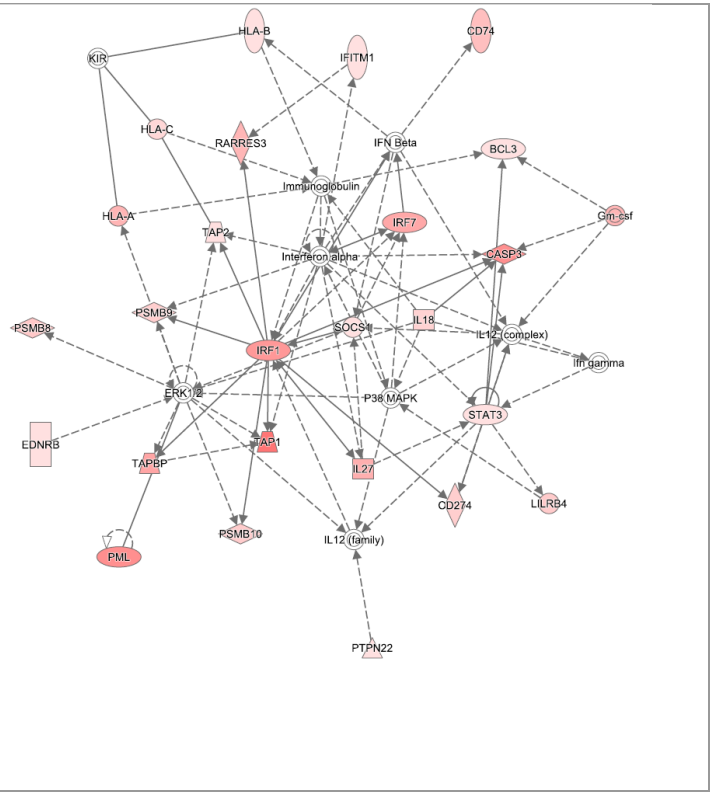
Stimulated LTBI vs LTBI-T2D	Antigen Presentation Pathway Th1 and Th2 Activation Pathway Th1 Pathway Crosstalk between Dendritic Cells and Natural Killer Cells Type I Diabetes Mellitus Signalling	Connective Tissue Disorders Immunological Disease Inflammatory Disease Inflammatory Response Organismal Injury and Abnormalities	Cellular Development Cellular Growth and Proliferation Cellular Function and Maintenance Cell Death and Survival Cell Cycle	Haematological System Development and Function Haematopoiesis Lymphoid Tissue Structure and Development Tissue Development Cell-mediated Immune Response	

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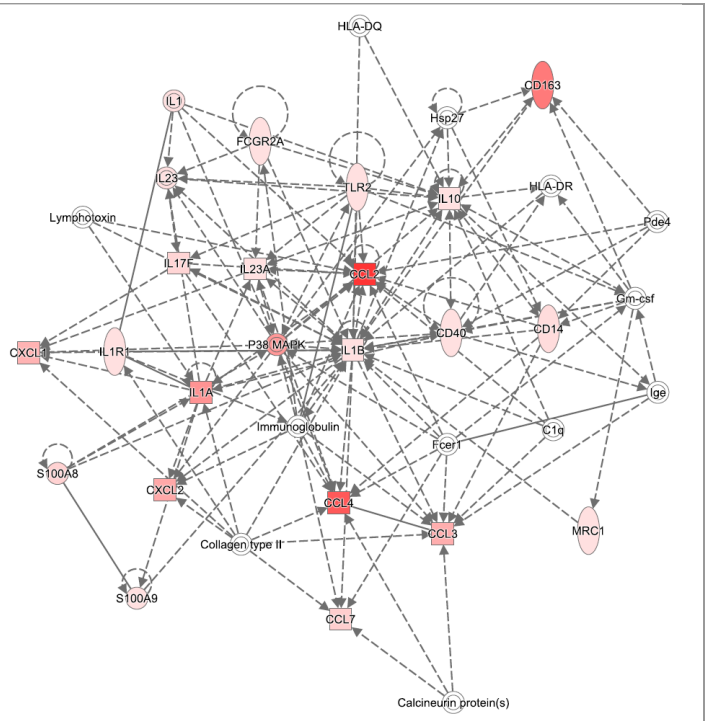
Unstimulated TB vs TB-T2D, BL	TREM1 Signalling Differential Regulation of Cytokine Production in Intestinal Epithelial Cells by IL-17A and IL-17F Differential Regulation of Cytokine Production in Macrophages and T Helper Cells by IL-17A and IL-17F Hepatic Fibrosis / Hepatic Stellate Cell Activation Dendritic Cell Maturation	Infectious Diseases Immunological Disease Inflammatory Response Connective Tissue Disorders Inflammatory Disease	Cell-To-Cell Signalling and Interaction Cell Death and Survival Cellular Movement Cellular Function and Maintenance Cellular Development	Haematological System Development and Function Immune Cell Trafficking Haematopoiesis Lymphoid Tissue Structure and Development Tissue Development	

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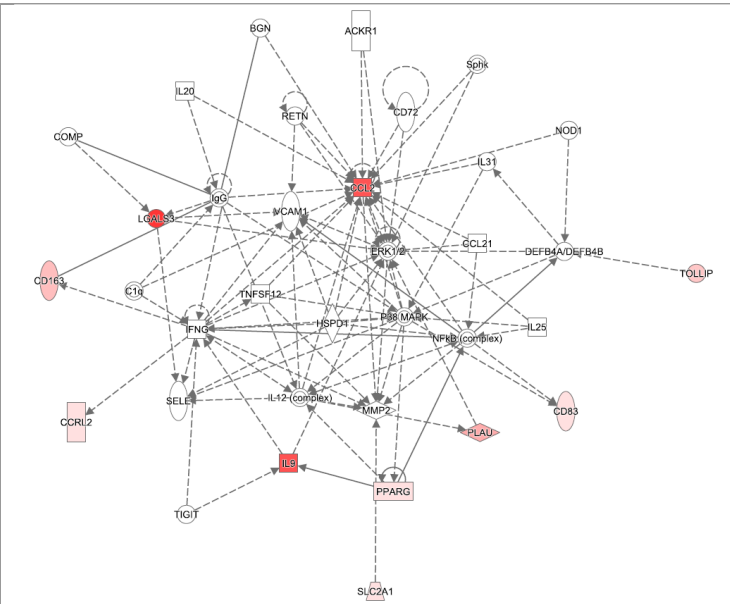
Stimulated TB vs TB-T2D, BL	Differential Regulation of Cytokine Production in Macrophages and T Helper Cells by IL-17A and IL-17F Differential Regulation of Cytokine Production in Intestinal Epithelial Cells by IL-17A and IL-17F Role of Hypercytokinemia/hyperchemokinemia in the Pathogenesis of Influenza TREM1 Signalling Glucocorticoid Receptor Signalling	Hypersensitivity Response Inflammatory Response Connective Tissue Disorders Organismal Injury and Abnormalities Skeletal and Muscular Disorders	Cell-To-Cell Signalling and Interaction Cellular Movement Cell Death and Survival Cellular Compromise Cellular Function and Maintenance	Haematological System Development and Function Immune Cell Trafficking Tissue Morphology Connective Tissue Development and Function Tissue Development	

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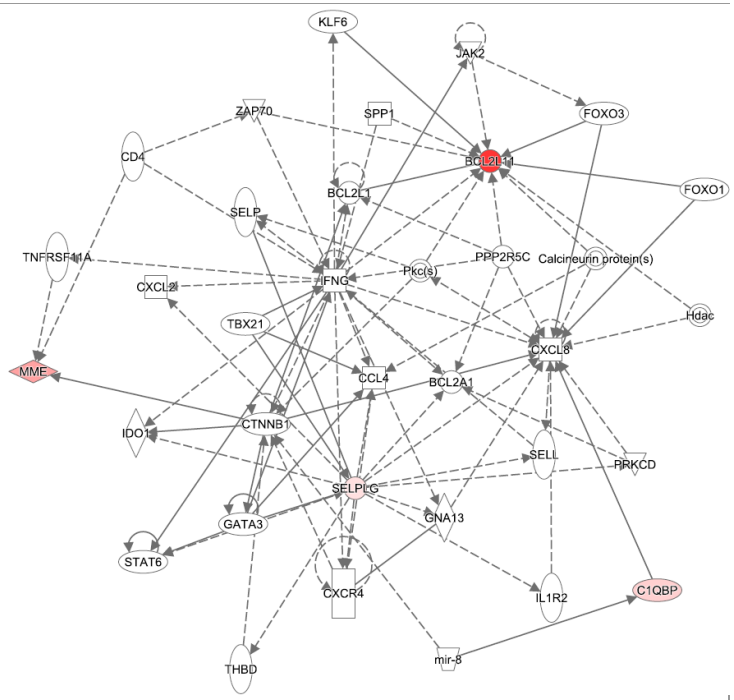
Unstimulated TB vs TB-THG, BL	Complement System Apoptosis Signalling Neuroprotective Role of THOP1 in Alzheimer's Disease Atherosclerosis Signalling PTEN Signalling	Endocrine System Disorders Gastrointestinal Disease Metabolic Disease Organismal Injury and Abnormalities Cancer	Cell-To-Cell Signalling and Interaction Cell Death and Survival Cell Morphology Cellular Assembly and Organization Cellular Function and Maintenance	Cardiovascular System Development and Function Cell-mediated Immune Response Embryonic Development Haematological System Development and Function Haematopoiesis	

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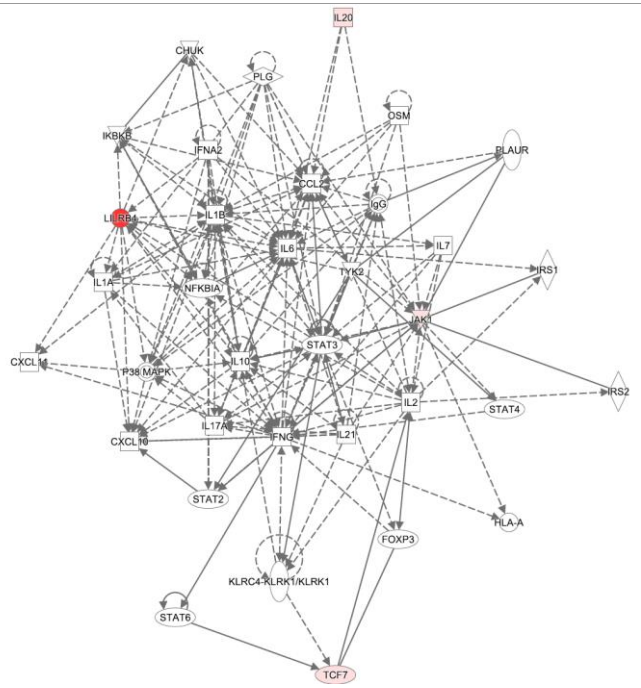
Stimulated TB vs TB-THG, BL	IL-22 Signalling Role of JAK1, JAK2 and TYK2 in Interferon Signalling Role of JAK family kinases in IL-6-type Cytokine Signalling IL-15 Production Role of JAK2 in Hormone-like Cytokine Signalling	Connective Tissue Disorders Dermatological Diseases and Conditions Inflammatory Disease Inflammatory Response Organismal Injury and Abnormalities	Cellular Development Cellular Function and Maintenance Cellular Growth and Proliferation Cell-To-Cell signalling and Interaction Cellular Movement	Cell-mediated Immune Response Embryonic Development Haematological System Development and Function Haematopoiesis Lymphoid Tissue Structure and Development	

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3.4. Discussion

Humans infected with Mtb can clear the pathogen, become latently infected or develop active TB disease (Lu et al. 2011) and the risk of progression is significantly increased in T2D patients (Jeon and Murray, 2008). However, the factors influencing pathogen clearance and disease progression from latent infection are poorly understood (O'Garra et al. 2013). Our study investigated gene expression data from unstimulated and Mtb-antigen stimulated whole blood to identify distinct gene signatures in individuals with LTBI with and without T2D, and TB with and without T2D. We identified differential gene expression profiles, which may contribute to increased susceptibility of T2D patients to TB and increased disease severity in TB-T2D comorbidity, as well as delayed treatment response and unfavourable treatment outcomes.

T2D is a risk factor for poor TB treatment outcomes including treatment failure and relapse (Dooley et al. 2009; Critchely et al. 2017) and the WHO recommends that all active TB patients be screened for T2D. This is however not broadly implemented. The TB-THG group is characterised by high HbA1c prior to TB treatment which decreases during the first three months of treatment (Tabarsi et al. 2014). It is therefore advised to monitor HbA1c of TB patients during treatment in order to confirm their T2D status. Our study was able to identify a signature that could differentiate between TB-THG and TB-T2D, which could be useful in preventing the unnecessary treatment in those who would have been incorrectly diagnosed as having T2D. Most importantly, T2D screening of TB patients is recommended by the WHO (Restrepo et al. 2011), however HbA1c testing using a cut-off of 6.5% will not be able to distinguish patients with THG from T2D. Therefore, our gene signature could complement standard T2D screening of TB patients and also support a re-evaluation of HbA1c cut-offs for T2D diagnosis in TB patients.

We identified 15 gene transcripts that were dysregulated in unstimulated whole blood from TB-T2D patients compared to TB and TB-THG. It is possible that the products of these 15 gene transcripts (CCL2, CCL4, CCL3, IL-1A, CD163, CXCL2, PLAU, CXCL1, IL-1R1, TNFRSF8, CCRL2, NFKB1, PSMD7, PSMB5 and BAX) contribute to immunopathology in TB-T2D because their expression was unique to TB-T2D. Our unpublished data revealed that serum CCL2 protein is highly upregulated in TB-T2D patients, and could mean that high CCL2 in TB-T2D negatively feeds back to downregulation of CCL2 at the mRNA level. It is also possible however that the high CCL2 concentrations in the serum are a spill-over from the site of disease and produced by immune cells recruited to the lung and not by immune cells in the circulation. Others also showed that serum of TB-T2D patients have elevated CCL2 protein levels (Panee, 2013). It has been shown that downregulated proteins are well correlated with their mRNA gene transcripts, however this trend was not reported for upregulated proteins, because they were either correlated with unchanged or downregulated mRNA gene

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expression (Fournier et al. 2010). This could be why we observe downregulated CCL2 transcripts while its protein has been reported to be upregulated in TB-T2D patients (Panee, 2013). Insufficient CCL2 mRNA expression in diabetic wounds were reported to be primarily responsible for the delay in macrophage response in these wounds (Wood et al. 2014). We believe that the downregulation of the products of the gene transcripts mentioned above may favour altered recruitment of immune cells to the site of inflammation and affect immunity. Our results show that the TNFRSF8, BAX and NF-kB1 gene transcripts which are important for triggering apoptosis are inhibited in TB-T2D patients (Donath et al. 2003). Furthermore, Mitogen-Activated Protein Kinase-Activated Protein Kinase 2 (MAPKAPK2), a protein kinase downstream of the p38 mitogen-activated protein kinase pathway, is involved in pathological conditions by upregulating various inflammatory pathways (Fiore et al. 2015). MAPKAPK2 was found to be upregulated in TB-T2D compared to TB and TB-THG patients, in our study, and could be involved in promoting dysregulated inflammatory processes that are seen in TB-T2D patients. Complement system C1q transcript is highly expressed in early stages of TB disease (Esmail et al. 2018). Interestingly, compliment c1q binding protein (C1QBP) was downregulated in our unstimulated sample of TB-THG (vs TB). C1QBP was also downregulated after stimulation in T2D compared to LTBI. C1QBP and SERPING, also downregulated in T2D compared to LTBI, were highly abundant in whole blood of TB patients compared with LTBI (Esmail et al. 2018). Since the complement pathway is upregulated during Mtb infection and gets downregulated during TB treatment (Cliff et al. 2013), Suliman et al. speculated that c1q is involved in TB pathology (Suliman; Thompson et al. 2018). In keeping with this, its expression is strongly associated with active TB disease and disease severity (Cai et al. 2014). Even though the exact role of C1QBP is still unknown, it has been shown to play a role in regulating apoptosis and inflammatory processes in the mitochondrial matrix (Feichtinger et al. 2017). The downregulation of this transcript in our results was seen along with the downregulation of BCL2L11, which is an apoptotic gene. The inhibition of BCL2L11 is associated with inhibition of apoptosis as well as autophagy (Dai and Grant, 2015). In our unstimulated blood from TB-T2D patients compared to TB, BCL2L11 gene transcript was downregulated.

Immunity to TB requires effective innate and adaptive immune responses (Cooper and Khader, 2008; Beisiegel et al. 2009). NF-kB is a mediator of pro-inflammatory gene development and functions in both innate and adaptive immunity (Liu et al. 2017). NF-kB induction was high in TB compared to TB-T2D, this was accompanied by the induction of CD14 found on monocytes, CD163 expressed on macrophages and natural killer cell receptor 2B4 (CD244) which are important players of the innate immune response. As part of this study, in patients with TB compared to TB-T2D, the components contained in cytotoxic granules such

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as perforin (PRF1), granulysin (GNLY) and granzyme B (GZMB) were also elevated and corresponded to the expression of CD244 in unstimulated samples. Kumar et al. showed that the frequency of $CD8^+$ T cells producing PRF1 or GZMB or CD107a were decreased in TB-T2D group compared to TB in unstimulated whole blood and even after stimulation with Mtb-antigens (CFP-10 or ESAT-6) (Kumar et al. 2015). NK cells producing PRF1 or GZMB were not significantly different between TB-T2D and TB at baseline and after stimulation with Mtb-antigens, however, CD107a produced by NK cells was significantly lower in TB-T2D compared to TB after Mtb-antigens stimulation (Kumar et al. 2015). Kumar's finding is different from our gene expression results since we saw similar results in the unstimulated blood and not after Mtb-antigens stimulation. However, this is not surprising since the gene expression and its protein abundance has been shown to be poorly correlated (Maier et al. 2009). The key transcription factor NF-kB of M1 macrophages is essential for the development of the Th1 responses such as IL-12, IFN-γ, IL-23, and IL-27 which are required to control Mtb replication (Cooper and Khader, 2008), as well as IL-1β, TNF-α, and IL-6 (Wang et al. 2014). Blood transcriptional assays that enable early accurate TB diagnosis could have an important impact on control of the global TB epidemic (Walter et al. 2016). The immunological factors involved in the host immune response network in Mtb infection and TB reactivation have not yet been clearly elucidated. Identifying the host genes responsible for TB susceptibility and resistance may be the key towards better understanding the mechanisms leading to the development of the disease, and also identify pathways for targeted HDT. The genes that were differentially expressed in unstimulated LTBI-T2D compared to LTBI were associated with activation of necrosis and cell death of tumour cells, inhibition of immune response of leukocytes, maturation of blood cells, which may be implicated in the regulation of the immune response during Mtb infection. CCL2, CD163, TNFRSF8, cytokine inducible SH2 containing protein (CISH), guanylate binding protein 5(GBP5), promyelocytic leukemia (PML) and transporter associated with antigen presentation (TAP1) were differentially expressed in LTBI-T2D compared to LTBI and TB-T2D compared to TB patients which implies that these genes could have some significance in promoting TB susceptibility in LTBI and pathology in TB patients with T2D.

A gene signature unique to LTBI-T2D patients was identified and includes CD59, TNFS11 and CD274. Only CD59 was significantly higher in individuals with LTBI-T2D compared to LTBI. CD59 is a membrane glycoprotein which protects host cells from the membrane attach complex mediating lysis (Koski et al. 1996). The membrane attack complex can result in tissue injury during dysregulated complement activation that occurs during chronic inflammatory conditions (Huang et al. 2006). This can result in overexpression of CD59, which, in tumour cells has been associated with tumour development (Fishelson et al. 2003). In our LTBI-T2D

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patients, it is possible that CD59 protects the Mtb infected cells from complement mediated lysis which favours replication within the cells. A process which is perhaps exploited by Mtb.

Zak et al. identified a 16 gene signature that could predict development of TB two years prior to diagnosis (Zak et al. 2016). Suliman et al. also identified a RISK4 gene signature that identifies those at risk for TB progression within two years (Suliman et al. 2018). In our study, TAP1, SERPING-1 and GBP5 genes were downregulated in T2D compared to LTBI after stimulation with Mtb antigens. The mentioned genes are also in the 16 gene signature identified by Zak et al. Before Mtb antigens stimulation, these genes were upregulated in LTBI-T2D compared to LTBI, and were downregulated after stimulation. This shows that LTBI-T2D patients express some of the signature genes associated with progression to TB. Whether this is associated with an increased risk to progress to TB in T2D patients is not yet known. The complete signatures identified by Zak et al. (2016) and Suliman et al. (2018) could not be detected in our study. This was expected since a published 393-gene TB signature identified previously could not identify TB in patients co-infected with HIV (Dawany et al. 2014). The other reason was that our study used a specific immunological panel, which only looked at a subset of genes unlike the mentioned studies which used more in depth techniques such as RNA-seq. Due to T2D, the expression of genes is likely to differ.

The IL-18 gene transcript was downregulated in the RNA isolated from unstimulated blood and upregulated in the Mtb antigen stimulated blood of individuals with LTBI-T2D compared to LTBI. In our unpublished data, we showed higher serum protein levels of IL-18 in LTBI-T2D compared to LTBI. This means that there is an inverse relationship between IL-18 protein and mRNA (similarly to what we observed for CCL2), which could feedback to suppress the IL-18 transcript. In a study where IL-18 knock out mice were infected with Mtb, the IL-18 knock out mice had more CFUs in their lungs, compared to wildtype mice, and the CFUs were reduced after treating the mice with IL-18 (Sugawara et al 1999). In this study, the authors concluded that IL-18 is important for the development of protective immunity during Mtb infection (Sugawara et al. 1999). IL-18 increases FASL expression and NF-κB activation and its downregulation has been associated with increased Mtb susceptibility (Dinarello, 1999). The IL-18 gene has been studied in TB and LTBI models. We found it to be differentially expressed in LTBI-T2D compared to LTBI. Interestingly, in unstimulated LTBI-T2D samples, this gene transcript was downregulated and upregulated after Mtb antigen stimulation. This could be as a means of immune activation, and implies that IL-18 expression is affected during LTBI-T2D. These results have not been reported elsewhere, however, the inflammatory gene profile described by Zak et al. included this marker (Zak et al. 2016).

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Transforming growth factor beta (TGF-β) is an anti-inflammatory and immunosuppressive cytokine, it antagonizes the activation of NF-kB in lymphocytes, on the one hand, NF-kB also inhibit activities of TGF-β (Geiser et al. 1993). TGF-β receptor-1 transcript was increased in our results in the TB-T2D compared to TB, and we think that this increase could underlie or result in irregular TGF-β production. If it does influence TGF-β production, it could explain the increased Th2 responses that have been reported in other studies (Al-Attiya and Mustafa, 2009; Wang et al. 2018). However, in LTBI-T2D the upregulation of TGF- β is associated with the down regulation of Th1 (Kumar et al. 2014).

Generally, the immune responses (especially the NF-kB signalling pathway) against Mtb were downregulated in the unstimulated blood from TB-T2D patients which could be a contributing factor in delayed Mtb clearance and unfavourable TB treatment outcomes. Interestingly, TNFRSF8 or CD30 which is expressed by activated lymphocytes (T and B cells), was downregulated in TB-T2D and one of its major functions is to arbitrate the signal transduction that leads to the activation of NF-kB (Liu et al. 2017). TNFRSF8 was also down-regulated in unstimulated LTBI-T2D compared to LTBI and as we know, TNF-α suppressors have been associated with progression to TB disease in LTBI individuals (Cantini et al. 2016).

We were able to perform gene expression assay on both TANDEM and ALERT samples and found that there are unique gene profiles among our study patients. These gene profiles will be useful to characterize the biological composition and the immune response profile during different disease states. We identified a 15 gene signature that was downregulated in TB-T2D patients when compared to both TB and TB-THG and suggested that it could be involved in the immunopathology associated with TB-T2D. In addition, we also identified a biosignature using RPA (C1QBP, C1R and PSMB5) that could distinguish between TB-THG and TB-T2D in unstimulated blood. We were able to identify novel gene signature in unstimulated blood (CD59, TNFSF11 and CD247) from individuals with LTBI-T2D compared to LTBI which should be further evaluated in functional assays to elucidate their involvement in the increased susceptibility to TB observed in individuals with T2D. We were not able to perform a direct comparison for our 594 gene expression between the participant groups of the two different studies as the gene expression analysis was batched and run at different times. The first gene expression analysis was performed on the TANDEM patients, and a year later we ran the same gene expression panel on ALERT patients. We further acknowledge that we had a relatively low sample size for the gene expression assays.

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CHAPTER 4

Immune-endocrine changes in the serum and plasma of individuals with LTBI and TB patients with and without T2D, association with glycaemic control and Mtb control in phagocytic mononuclear cells.

4.1 Introduction

T2D is increasing in low and middle income countries where TB is also endemic (Harries et al. 2011). T2D is considered a risk factor for TB; apart from hyperglycaemia, altered phagocytosis by monocytes, dysregulated T cell immune response, altered hormone and cytokine production have been reported in TB-T2D patients (Restrepo and Schlesinger, 2013; Gomez et al. 2013; Kumar et al. 2014). Individuals with LTBI-T2D exhibit diminished circulating levels of type 1 (IFN-γ, IL-2 and TNF-α) and type 17 (IL-17F) cytokines (Kumar et al. 2014). Furthermore, decreased systemic levels of other pro-inflammatory cytokines such as IL-1β and IL-18, and the anti-inflammatory cytokine IL-10 have been reported (Kumar et al. 2014). Moreover, inflammatory cytokine production can activate the HPA axis (Fernandez et al. 2016). The activation of the HPA axis leads to secretion of ACTH, ultimately resulting in the production of cortisol from the adrenals (Burford et al. 2017). Dysregulated cortisol production is associated with inhibition of immune cell functioning, such as antigen presentation leading to reduced Mtb control (Angerami et al. 2013), and has also been associated with TB severity (Santucci et al. 2011). The immune-endocrine profile is dysregulated in TB and more so in TB-T2D comorbidity (Fernandez et al. 2016). Little is known about the dysregulation of the immune-endocrine interaction in LTBI-T2D, although this may contribute to the susceptibility of T2D patients to TB disease. We therefore aimed to characterize cytokine and hormone levels in latently infected individuals and TB patients with and without T2D and evaluate if there is correlation among the cytokines, hormones and Mtb association and control by PBMCs and MNs from these participants.

4.2 Methods

The detailed methods are discussed in Chapter 2. Forty close contacts of TB patients with and without T2D (enrolled as part of the ALERT study) and 21 TB patients with and without T2D at BL and M6 (enrolled as part of the TANDEM study) were included. The blood collected in NaHep tubes was used for PBMC isolation using the density gradient centrifugation method (for ALERT patients). Subsequently, MNs were isolated using Miltenyi Pan Monocyte isolation kit and LS MACS separation columns (Miltenyi biotec). The PBMCs and MNs were infected with *H37Rv* Mtb for two hours. Mtb uptake was determined, and cells were further incubated for one and three days (for MNs) or six days (for PBMCs), to determine the ability of the MNs and PBMCs to control and kill the Mtb in both the LTBI (noT2D) and LTBI-T2D (T2D). Differential blood cell counts and clinical parameters (HbA1c, FBG, lipids, weight and height) were determined. Weight and height was used to calculate the BMI of each participant. The

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serum and plasma samples that were previously stored at -80°C were thawed and ELISAs used to measure adrenaline, noradrenaline, dopamine, (Abnova) serotonin, I-TAC and IL-22 (Biocom). Luminex was used to simultaneously measure IFN-γ, TNF-α, IL-1β, IL-4, IL-6, IL-8, IL-10, IL-13, IIL-18, IL-21, IL-22, IL-23, IL-27, IL-33, (Qiagen), IL-35 and oncostatin (OSM) (Merck-Millipore) in the same samples. The CFU data were correlated with the cytokines, hormones, blood glucose levels, lipids and differential blood cell counts. IL-22 ELISA was performed on serum samples from both the ALERT and TANDEM study. Repeated measures ANOVA with a Fisher LSD *post hoc* test as well as Kruskal-Wallis with Dunn's *post hoc* test were used to determine statistical differences between the groups. Spearman correlation analysis was used to determine association between variables.

4.3 Results

4.3.1 Study participants and characteristics

The participants included in this chapter are the same as in the previous chapter. Their demographics and clinical parameters are summarized in Table 3.1. The ALERT participants were latently infected individuals with and without T2D, and in this particular chapter, participants were divided into four subclasses; noT2D (HbA1c 4.0-5.69%, n=10), PreT2D (HbA1c 5.7-6.49%, n=10), T2D (HbA1c 6.5-7.9%, n= 10) and poorly controlled T2D (pT2D) (HbA1c ≥8%, n=10). TANDEM participants were TB patients with and without T2D, and were grouped as follow, TB (HbA1c ≤5.7%), TB with transient hyperglycaemia (TB-THG; HbA1c ≥6.5% at BL only and not on DM medication) and TB-T2D (HbA1c ≥6.5% at BL and M6, and taking DM medication).

4.3.2 Cytokine responses in latently infected individuals with and without T2D

T2D is characterised by chronic inflammation and dysregulated immune response (Xia et al. 2017). LTBI-T2D have low circulating levels of IFN-γ, IL-2, TNF-α and IL-17F (Kumar et al. 2014), as well as IL-1β, IL-18, IL-10 (Kumar et al. 2014). In this study, we measured the circulating concentrations of Th1 (IFN-γ and TNF-α), Th2 (IL-4, IL-10, IL-13, IL-33, IL-35 and IL-27), Th17 (IL-22) as well as a range of other pro-inflammatory cytokines (IL-1β, IL-6, IL-18, IL-21 and IL-23) and chemokines (IL-8 and I-TAC) in the serum and plasma of individuals with LTBI with and without T2D.

In this section we first compared the cytokine concentrations in the two groups, LTBI (including noT2D and preT2D) and LTBI-T2D (includingT2D and pT2Ds). Serum concentrations of IL-6 were significantly higher in individuals with LTBI-T2D compared to LTBI-noT2D (Figure 4.1A). IL-18 showed a trend towards higher concentrations in LTBI-T2D compared to LTBI-noT2D (Figure 4.1B). There was no significant difference in concentrations of the following cytokines; IL-27 (Figure 4.1C), IL-4, IL-8, IL-13, IL-22, I-TAC and OSM (data not shown) between LTBI-

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T2D and LTBI-noT2D. IFN-γ, TNF-α, IL-10, IL- 21, IL-22, IL-23, IL-33 and IL-35 were not detected in majority of the study participants and therefore were not included in the analysis.

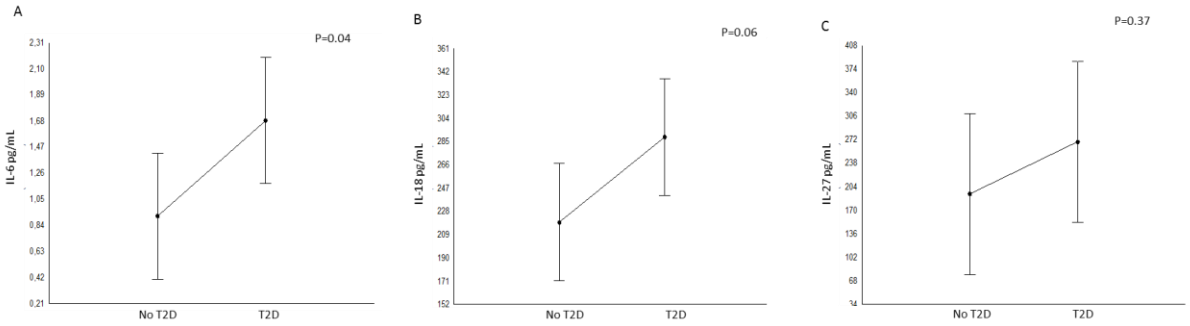

Figure 4.1 Circulating cytokine levels in LTBI-noT2D compared to LTBI-T2D. IFN-γ, TNF-α, IL-1β, IL-4, IL-6, IL-8, IL-10, IL-13, IL-18, IL-21, IL-22, IL-23, IL-27, IL-33, IL-35, OSM were measured using luminex and I-TAC and IL-22 using ELISA in serum samples of LTBI-noT2D (HbA1c 4.0-6.4%, n=20) and LTBI-T2D (HbA1c ≥6.5%, n=20) patients. (A) IL-6 concentrations were significantly higher in LTBI-T2D compared to LTBI-noT2D. (B) IL-18 concentrations were also higher in LTBI-T2D patients compared to LTBI-noT2D participants, but did not reach statistical significance. (C) IL-27 concentrations were not significantly different between LTBI-noT2D and LTBI-T2D. Data are represented as LS means with 95% confidence intervals (CI). The significant difference between the groups was calculated using the Mann-Whitney U T test and a p-value < 0.05 was considered significant.

4.3.3 IL-22 and IL-18 concentrations are higher in pT2D patients

IL-22 has been shown to induce antimicrobial peptides, promotes epithelial repair and inhibits intracellular growth of Mtb in macrophages (reviewed in Domingo-gonzalez et al. 2016). IL-18, produced by monocytes and macrophages, is associated with protection against Mtb infection (Schneider et al. 2010). When further subdividing the study participant into LTBI with preT2D, T2D and pT2D, we found that IL-18 concentrations were significantly higher in LTBI-pT2D compared to LTBI-noT2D (p=0.02; Figure 4.2A). There was a trend towards higher levels of IL-18 in LTBI-pT2D when compared to LTBI-preT2D (p=0.06; Figure 4.2A). IL-22 was significantly higher in LTBI-pT2D compared to LTBI-T2D (p=0.04; Figure 4.2B). IL-22 was also higher in LTBI-pT2D compared to LTBI-noT2D, however, this difference failed to reach significant difference (p=0.09). IL-6 did not show any significant difference when comparing the four groups to each other.

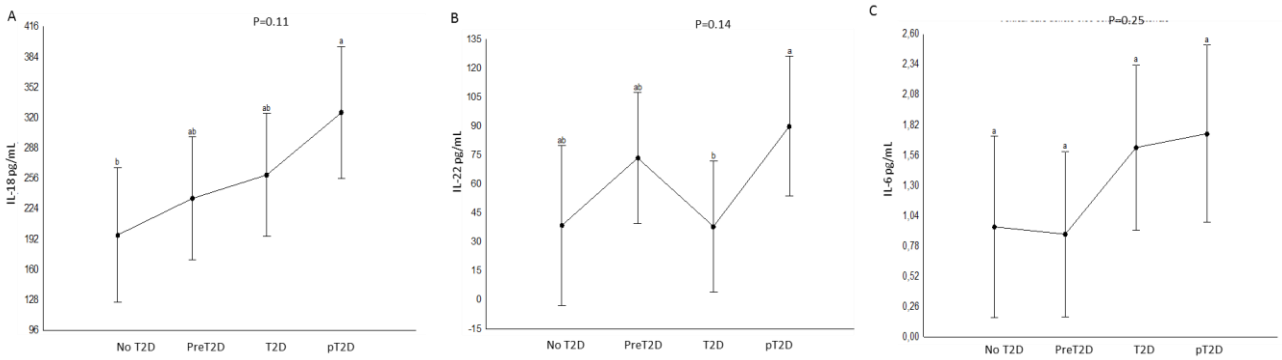

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Figure 4.2 IL-18, IL-22 and IL-6, concentrations compared among LTBI-noT2D, preT2D, T2D and LTBI-pT2D patients. Serum samples from LTBI-noT2D (HbA1c% 4.0-5.69, n=10), PreT2D (HbA1c% 5.7-6.49, n=10), T2D (HbA1c% 6.5-8, n= 10) and pT2D (HbA1c%≥8, n=10) were used to measure IFN-γ, TNF-α, IL-1β, IL-4, IL-6, IL-8, IL-10, IL-13, IL-18, IL-21, IL-22, IL-23, IL-27, IL-33, IL-35 and OSM using the luminex technology and IL-22 and I-TAC using ELISA. (A) IL-18 is significantly higher in LTBI-pT2D (vs noT2D). (B) IL-22 is higher in LTBI-pT2D compared to LTBI-T2D (p=0.02) and LTBI-preT2D (p=0.09). (C) No statistical significant difference in IL-6 is observed among the four groups. The data were analysed using an ANOVA with a Fisher LSD Post-hoc test. Data are represented as LS means with 95% CI, ANOVA p value is shown on the graph. Letters (a-b) are used to indicate significant differences, groups with the same letter are not statistically different from each other.

4.3.4 IL-22 cytokine is higher in TB-T2D

The role of IL-22 in TB is contradicting, with some studies suggesting a pathological role, while other studies suggest a protective role for IL-22 during TB (Treerat et al. 2017). Given the importance of IL-22, we measured its concentration in TB, TB-THG and TB-T2D patients at BL and M6 using an ELISA. IL-22 concentrations were significantly lower in TB-THG patients compared to TB-T2D at BL (Figure 4.3A) and this trend was also seen at M6, although not statistically significant (Figure 4.3B). To determine if TB treatment has an effect on IL-22 levels, we compared IL-22 expression at BL and M6 in the three groups and no differences were observed, however, TB-T2D showed a trend towards decreased IL-22 at M6 compared to BL (Figure 4.3C).

Furthermore, we wanted to compare the concentration of IL-22 in TB, TB-THG and TB-T2D patients before and after TB treatment to that of individuals with LTBI with and without T2D. There was an overall significant difference in IL-22 concentration at BL (ANOVA), however, after correcting for multiple comparisons with the Dunn's *post hoc* test, there were no significant differences detected among any of the groups (Figure 4.3D). When comparing the end of treatment (M6) IL-22 concentrations of the TB patients to those measured in the LTBI groups, we found that the end of treatment, IL-22 concentrations were significantly higher in the TB-T2D (M6) compared to LTBI and LTBI-T2D (T2D) (Figure 4.3E).

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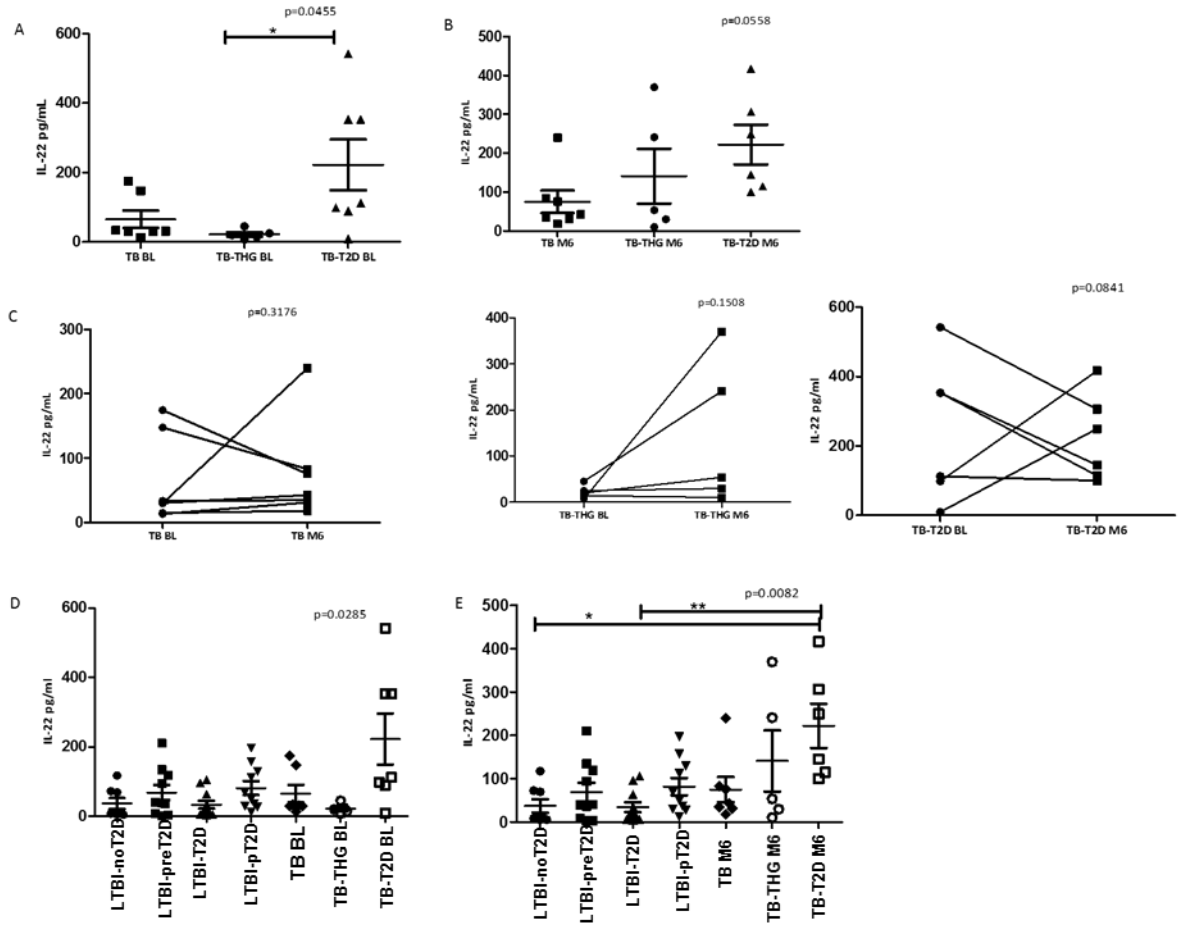

Figure 4.3 IL-22 concentrations in LTBI-T2D compared to TB-T2D. Serum from individuals with LTBI with noT2D (HbA1c 4.0-5.69%), preT2D (HbA1c 5.7-6.49%), T2D (HbA1c 6.5-8%) and pT2D (HbA1c ≥8%) was used for measuring IL-22 using ELISA. (A) IL-22 measurement in TB, TB-THG and TB-T2D at BL. (B) IL-22 measurement in TB, TB-THG and TB-T2D at M6. (C) IL-22 measurements to determine treatment effect in TB, TB-THG and TB-T2D at BL and M6. (D) IL-22 measurement in LTBI-noT2D, LTBI-preT2D, LTBI-T2D, LTBI-pT2D, TB, TB-THG and TB-T2D at BL. (E) IL-22 measurement in LTIB-noT2D, LTBI-preT2D, LTBI-T2D, LTBI-pT2D, and M6 of TB, TB-THG and TB-T2D. The ANOVA p-value is shown on the graph and differences between the groups indicated with a *. The data was analysed using the Kruskal-Wallis test with a Dunn's *post hoc* test to determine difference between the groups. *P <0.05, **P<0.01.

4.3.5 No differences in catecholamines, vitamin D, cortisol and insulin concentrations in LTBI participants with and without T2D

Cytokines produced during Mtb infection and T2D may alter endocrine responses which in turn could have an effect on theprogression of the disease (Webster et al. 2002). We therefore wanted to investigate the plasma concentrations of some hormones (mentioned below) in LTBI participants with and without T2D. We measured catecholamine (dopamine, adrenaline and noradrenaline) and serotonin concentrations with ELISA, while measurements of insulin, cortisol and Vitamin D levels were done at the national health laboratory services for these participants. We were unable to measure Noradrenaline due to technical problems encountered in the laboratory. No differences were observed in the circulating concentrations of the measured hormones between LTBI-T2D and LTBI-noT2D. Figures comparing the levels of cortisol (Figure 4.4A), insulin (Figure 4.4B), adrenaline (Figure 4.4C) and serotonin (Figure

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4.5D), between T2D and noT2D, are shown below. Dopamine and Vitamin D data were not included.

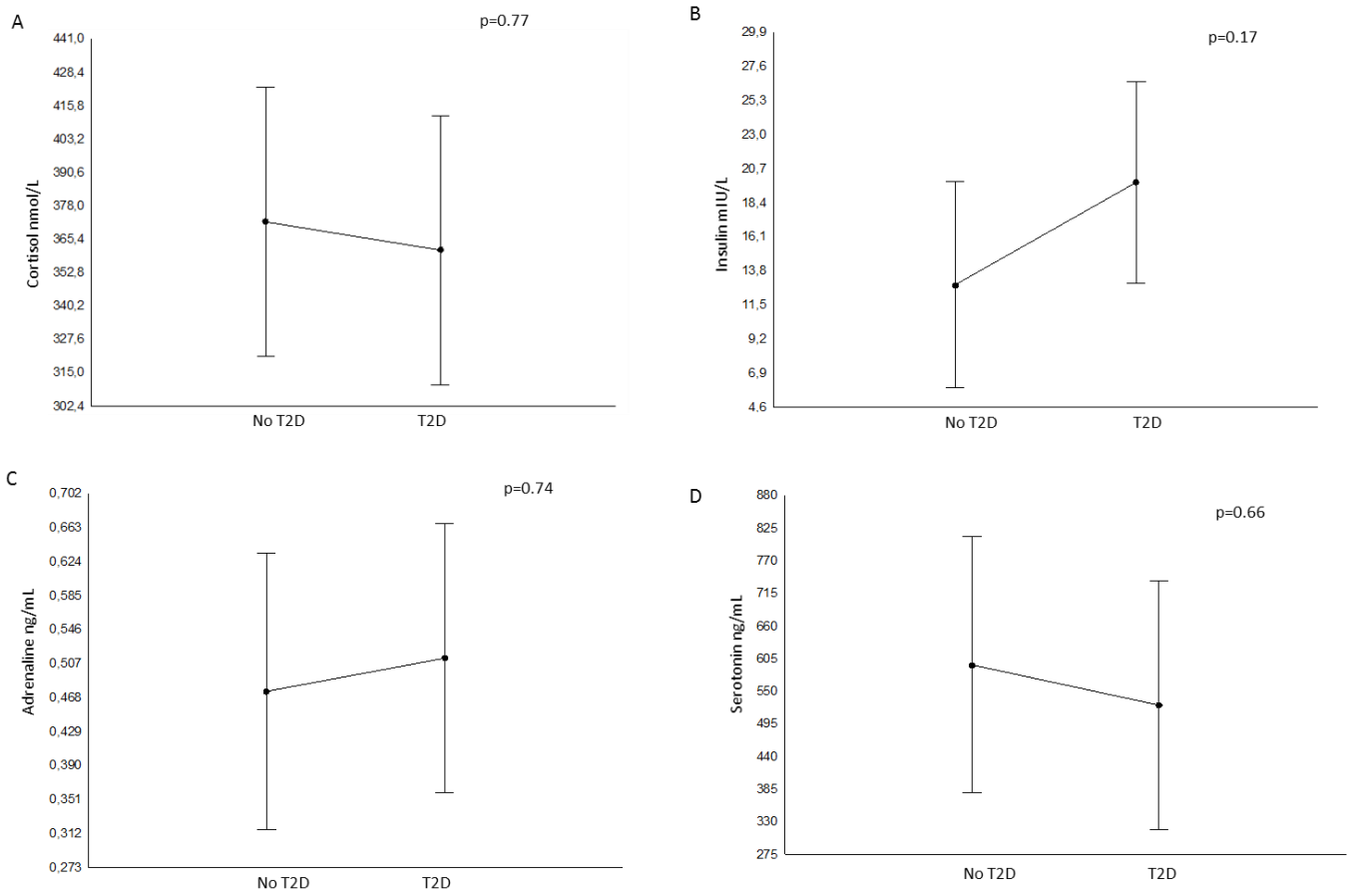

Figure 4.4 Hormone concentrations in LTBI-T2D compared to LTBI-noT2D. Measurements of cortisol (A) and insulin (B) were assessed by clinical laboratory testing through the national health laboratory services (NHLS). Adrenaline (C) and serotonin (D) were measured with ELISA. All the hormones were not significantly different between LTBI-T2D and LTBI-noT2D. Data was analysed using Mann-Whitney U T test and represented as LS means with 95% CI. P <0.05 was considered significant.

4.3.6 Insulin was higher in LTBI with pT2D compared to LTBI with noT2D

It is known that T2D patients are characterized by hyperglycaemia and insulin resistance (Taskinen and Boren, 2015), therefore we wanted to determine if subdividing our participants according to glycaemic levels would result in differences in hormones associated with T2D. After subdividing the participants into the four groups, there was a significant difference in insulin levels between LTBI-pT2D and LTBI-noT2D (p=0.03; Figure 4.5A). This is not surprising as high insulin observed in the LTBI-pT2D patients is driven by the insulin treatment some of the patients received (5 of the 10 pT2D patients reported using insulin) or could be due to hyperinsulineamia. There were no differences observed in any of the hormones; vitamin D, cortisol, serotonin and catecholamines measured when comparing the four patient groups (Fig 4.5B).

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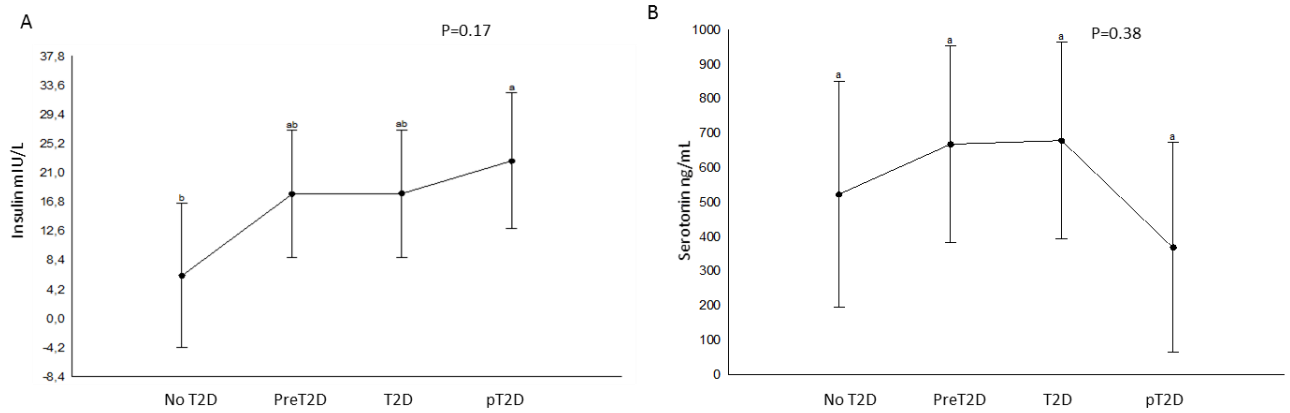

Figure 4.5. **Hormone levels in patients with LTBI and noT2D, preT2D, T2D and pT2D.** Insulin (A) and serotonin (B) concentrations were measure in the plasma samples of LTBI-noT2D (HbA1c 4.0-5.69%), LTBI-preT2D (HbA1c 5.7-5.64%), LTBI-T2D (HbA1c 6.5-8%) and LTBI-pT2D (HbA1c ≥8%) patients by ELISA. The data was analysed using an ANOVA with a Fisher LSD *post hoc* test. Data are represented as LS means with 95% CI. Letters (a-b) indicate significance differences. Groups with the same letter are not statistically different from each other.

4.3.7 Triglycerides were higher in LTBI-T2D compared to LTBI-noT2D

Dyslipidaemia is one of the best described characteristics of T2D (Taskinen and Boren, 2015), and is involved in the regulation of chronic inflammation (Wellen and Hotamisligil 2005). Since T2D is characterized by low grade chronic inflammation (Huang et al. 2014), we wanted to investigate the lipid profile in our study participants. We, therefore, investigated total CHL, triglycerides, LDL and HDL levels in the plasma of participants with LTBI with and without T2D. As shown in Figure 4.6A, the LTBI-T2D patients had significantly higher triglyceride concentrations in their blood compared to LTBI-noT2D. The total CHL (Figure 4.6B) and LDL (data not shown) were higher and HDL (data not shown) was lower in LTBI-T2D patients, but the differences were not significant.

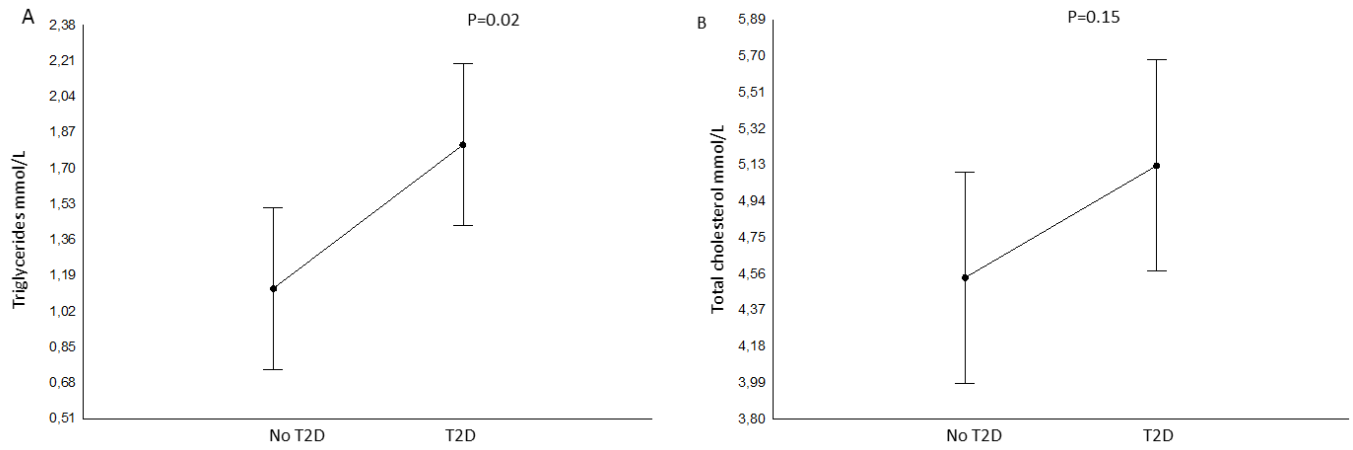

Figure 4.6 Triglycerides and cholesterol in LTBI-T2D compared to LTBI-noT2D. Lipids concentrations were assessed by clinical laboratory testing through the national laboratory services (NHLS). (A) Triglycerides were significantly higher in LTBI-T2D compared to LTBI-noT2D. (B) Total CHL was not significantly different between

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the two groups. Data were analysed using Mann-Whitney U T test. Data are represented as LS means with 95% CI. P <0.05 was considered significant.

4.3.8 Poorly controlled glycaemia drives the increase in lipids in T2D patients

To determine if the differences between the participants were dependant on the glycaemic control, the analysis was done in the four subgroups. Triglycerides were significantly higher in LTBI-pT2D compared to all the other groups (Figure 4.7A). Total CHL was significantly higher in LTBI-pT2D in comparison to LTBI-noT2D (p=0.01; Figure 4.7B). LDL was significantly higher in LTBI-pT2D compared to LTBI-noT2D (p=0.01; Figure 4C) and showed a trend towards higher levels compared to LTBI-preT2D (p=0.06; Figure 4.7C). HDL concentration was significantly higher in LTBI-noT2D compared to LTBI-PreT2D and LTBI-T2D (p=0.03; Figure 4.7D). Overall, all lipids measurements were higher in LTBI-pT2D compared to LTBI-noT2D, with the exception of HDL, which was the highest in the LTBI-noT2D.

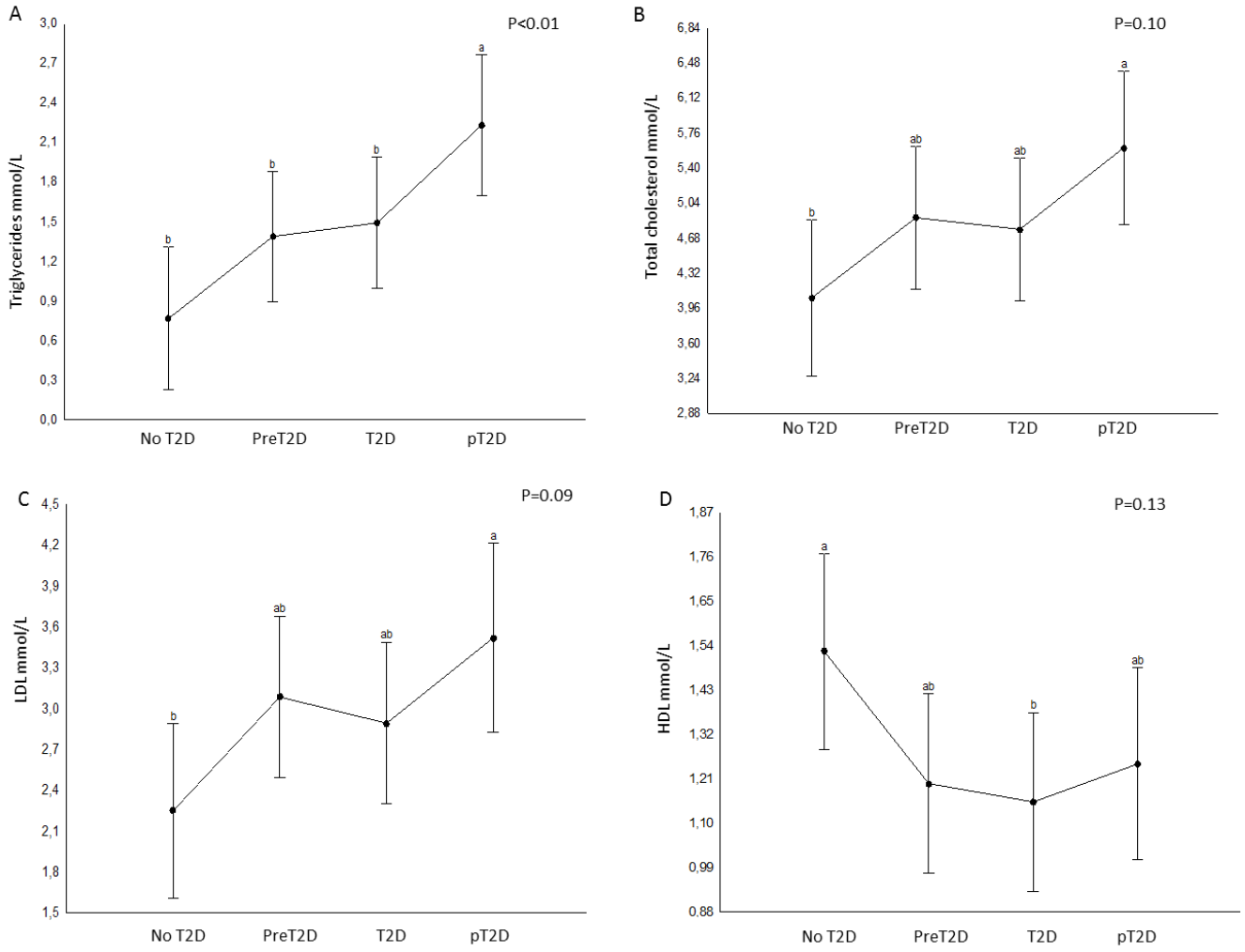

Figure 4.7 Lipid concentrations in LTBI-noT2D, LTBI-preT2D, LTBI-T2D and LTBI-pT2D participant groups. Lipids were measured in serum from LTBI-noT2D (HbA1c 4.0-5.69%, n=10), LTBI-PreT2D (HbA1c 5.7-6.49%, n=10), LTBI-T2D (HbA1c 6.5-8%, n= 10) and LTBI-pT2D (HbA1c ≥8%, n=10). (A) Triglycerides were significantly higher in LTBI-pT2D compared to the other groups. (B) Total CHL was significantly higher in LTBI-pT2D compared

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to LTBI-noT2D. (C) LDL was also significantly higher in LTB-pT2D (compared to LTBI-noT2D) and there was a trend for it to be higher in than LTBI-preT2D. (D) HDL was significantly lower in LTBI-noT2D (compared to LTBI-T2D). The obtained concentrations were analysed using an ANOVA with a Fisher LSD *post hoc* test. Data are represented as LS means with 95% CI. Letters (a-b) are used to indicate significance. Groups with the same letter are not statistically different from each other.

4.3.9 No difference in complete blood cell counts in LTBI with and without T2D

To determine the effect T2D might have on the full and differential blood cell counts, we compared the absolute number and frequency of blood cells in LTBI participants with and without T2D. Total red and white blood cells, as well as platelet counts were measured. In addition, neutrophil, monocyte, basophil and lymphocyte numbers were determined. No significant changes in the full and differential cell counts were observed between LTBI-T2D and LTBI-noT2D (data not shown).

4.3.10 Individuals with LTBI and pT2D have increased white blood cell and lymphocyte counts

Differential blood cell counts in our participants after stratification according to HbA1c was determined and we found higher white blood cell counts in LTBI-pT2D compared to LTBI-noT2D (p=0.01; Figure 4.8A) and a trend towards higher levels in LTBI-pT2D when compared to preT2D (p=0.08 Figure 4.8A). The lymphocyte counts were higher in the LTBI-pT2D compared to LTBI-noT2D (p=0.01), preT2D (p=0.02) and T2D (p=0.02) as shown in (Figure 4.8B). The neutrophil absolute counts did not show any differences, however, there was a trend towards higher counts in both LTBI-pT2D and LTBI-preT2D compared to LTBI-noT2D (p=0.07) (data not shown). There was no difference among the absolute counts of monocytes, eosinophils and basophils, and there was also no significant changes in the frequency of all the different blood cell counts among the four groups.

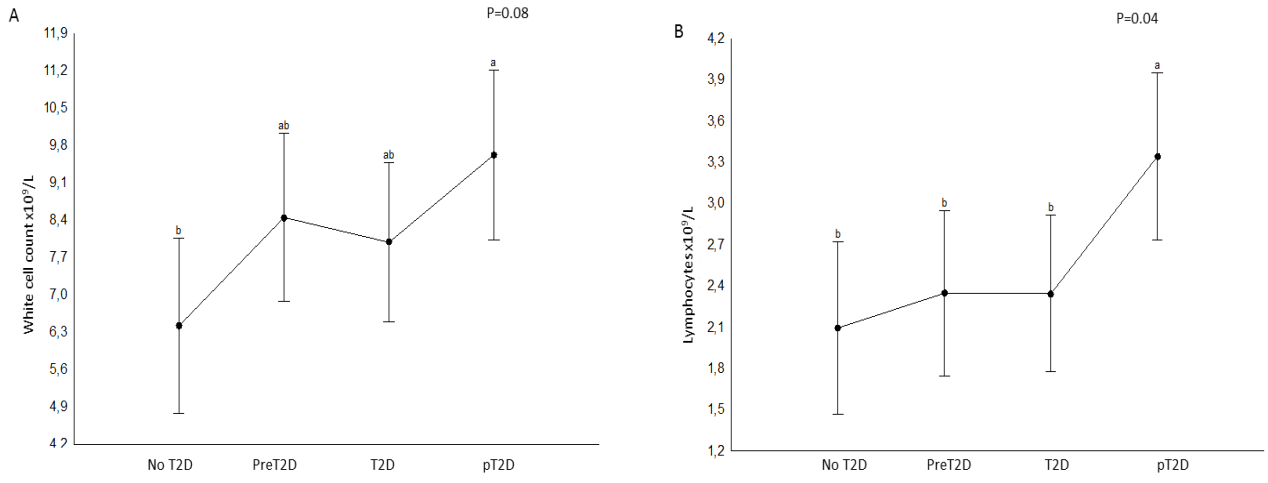

Figure 4.8. **Absolute counts of white blood cells and lymphocytes in participants with LTBI and noT2D, preT2D, T2D and pT2D.** Whole blood from LTBI-noT2D (HbA1c 4.0-5.69%, n=10), LTBI-preT2D (HbA1c 5.7-6.49%, n=10), LTBI-T2D (HbA1c 6.5-8%, n= 10) and LTBI-pT2D (HbA1c ≥8%, n=10) was used to measure the blood cell counts. (A) Illustrates significantly higher white blood cell count in LTBI-pT2D compared to LTBI-noT2D. (B) Lymphocyte counts are significantly higher in LTBI-pT2D compared to noT2D, preT2D and T2D. The obtained counts were analysed using an ANOVA with Fisher LSD *post hoc* test to determine difference between groups.

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Data are represented as LS means with 95% CI. Letters (a-b) are used to indicate significance. Groups with the same letter are not statistically different from each other. P <0.05 was considered significant.

4.3.11 PBMCs from participants with LTBI and T2D show poor association and control of Mtb

Innate responses are compromised in patients with chronic hyperglycaemia and macrophages from these patients have impaired association as well as bactericidal activity (Llorente et al. 2000). In this study, the PBMCs from individuals with LTBI with and without T2D were infected with Mtb *H37Rv* to determineassociation and killing activity. The CFU count at two hours was used as a measure of the number of Mtb associated with MNs, and the CFU counts at day one (D1), day three (D3) (MNs) or day six (D6) (PBMCs) as a measure of controlling Mtb growth, and decreasing CFU over time is a sign of Mtb killing. There was no correction made for the differential blood cell counts.

PBMCs from LTBI-T2D patients had significantly lower CFUs at two hours suggesting poorassociation of Mtb when compared to LTBI-noT2D (p=0.04; Figure 4.9A). Fold changes in CFU counts at D1 and D6 were calculated by dividing the CFU counts of each of these time points with the corresponding two-hour CFU counts. Fold changes were used to control for variations in Mtb uptake. PBMCs from LTBI-T2D and LTBI-noT2D had significantly higher CFU counts at D6 compared to D1 (p=0.01 and p=0.02, respectively; Figure 4.9B). No difference in killing ability of the PBMCs were observed when LTBI-T2D were compared to LTBI-noT2D. It is important to note that these findings vary to those of the entire ALERT cohort showing no difference in phagocytosis between the two groups and also showing significantly higher Mtb fold growth in LTBI-T2D compared to LTBI-noT2D. The differences are most likely due to the small number of participants included in this substudy. The analysis for the larger parent study is still ongoing.

It has been shown that pT2D increases TB risks (Leung et al. 2008). For that reason we further classified and analysed the data of the participants in the four subclasses depending on HbA1c. We found that after two hours the CFU counts from PBMCs isolated from LTBI-T2D were significantly reduced compared to LTBI-noT2D (p=0.02), LTBI-preT2D (p<0.01) and interestingly to LTBI-pT2D (p=0.01) (Figure 4.9C). There was a trend towards higher CFU in PBMCs of LTBI-T2D compared to PBMC from LTBI-noT2D, at D6 (p=0.06; Figure 4.9D). Furthermore, the four subclasses were investigated to determine Mtb containment. PBMCs from LTBI-noT2D (p=0.03), LTBI-preT2D (p=0.02) and LTBI-T2D (p<0.01) had a significant increase in CFUs from D1 to D6 (Figure 4.9D), whereas, a trend towards an increase from D1 to D6 was observed in LTBI-pT2D (p=0.08; figure 4.9D).

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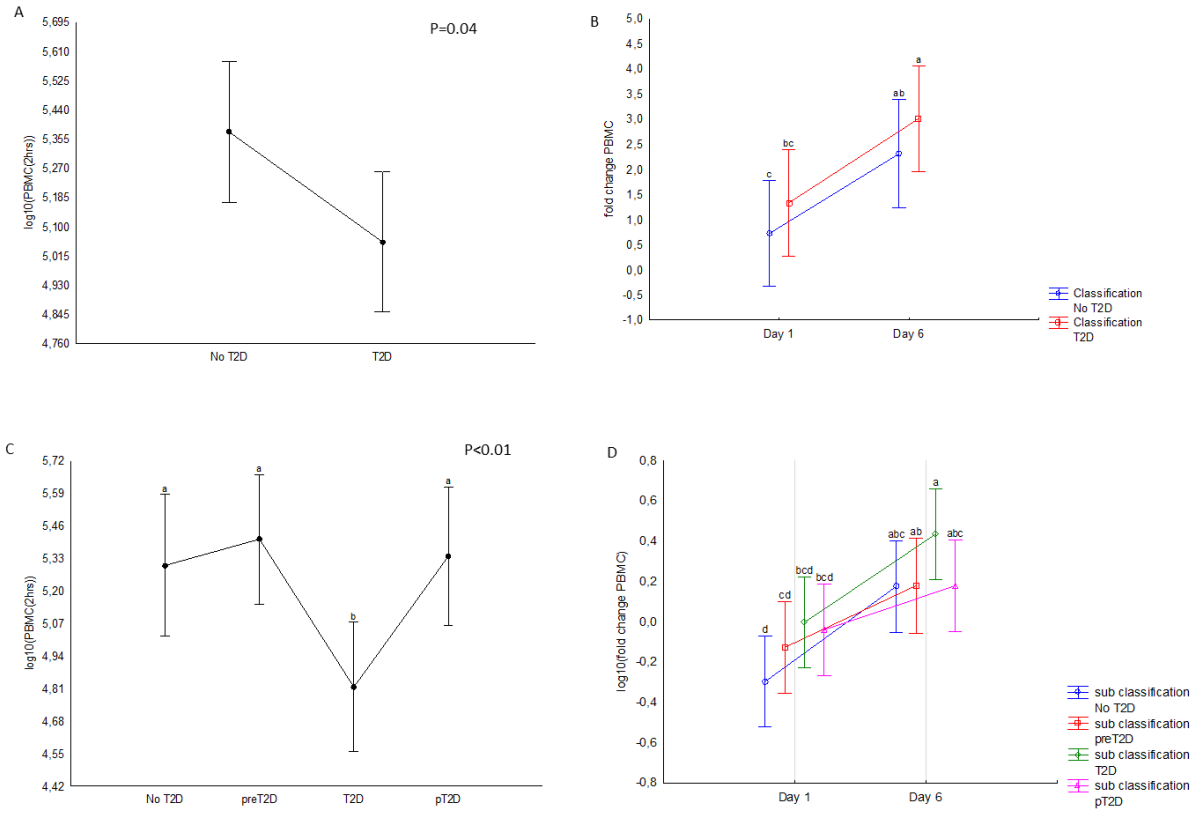

Figure 4.9 The influence of T2D on the containment of Mtb by PBMC of latently infected participants. PBMCs from LTBI-noT2D (HbA1c 5.7-6.4%, n=20) and LTBI-T2D (HbA1c ≥6.5%, n=20) (A and C) and PBMC from LTBI-noT2D (HbA1c 4.0-5.6%9, n=10), LTBI-PreT2D (HbA1c 5.7-6.49%, n=10), LTBI-T2D (HbA1c 6.5-8%, n= 10) and LTBI-pT2D (HbA1c≥8, n=10) (B and D) were infected with Mtb for two hours. Cells were lysed immediately after the infection as well as one and six days post infection. The data were analysed using ANOVA with a Fisher LSD *post hoc* test. Data are represented as LS means with 95% CI. Letters a-d indicate statistical significance with the same letter indicating no difference between or among groups.

4.3.12 MNs from T2D have poor Mtb control

MNs from patients with T2D have decreased phagocytic activity (Komura et al. 2010), and lower association with Mtb (Gomez et al. 2013) which could result in poor Mtb control. To determine the phagocytic capacity of MNs in our study cohort, MNs were isolated from PBMCs and infected with Mtb. Although our study showed that MNs from LTBI-noT2D had higher Mtb uptake compared to LTBI-T2D, the difference observed was not significant (Figure 4.10A). We further determined the Mtb killing capacity of the MNs from LTBI-noT2D and LTBI-T2D. CFUs increased in both groups from D1 to D3 with D3 CFU counts of LTBI-T2D reaching statistical significance (p=0.02; Figure 4.10B). In LTBI-noT2D the increase in CFUs from D1 to D3 remained a trend (p=0.06; Figure 4.10B). There was no significant difference in CFU between LTBI-T2D and LTBI-noT2D at D1 (p=0.47) and D3 (p=0.24) post infection (Figure 4.10B).

We further analysed the MNs CFU results according to the four subclasses and did not see any differences in the association of Mtb with MNs (Figure 4.10C). We continued to determine the growth and the ability of the MNs to control Mtb in these patients. Fold changes from D1 and D3 were compared. MNs from both LTBI-noT2D and LTBI-T2D showed a significant

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increase in CFU from D1 to D3 (p=0.04 and p<0.01, respectively; Figure 4.10D). The CFU fold growth from MNs of LTBI-preT2D and LTBI-pT2D did not significantly change from D1 to D3 (Figure 4.10D). There was no significant difference in CFU among the four groups at D1 and D3 (Figure 4.10D).

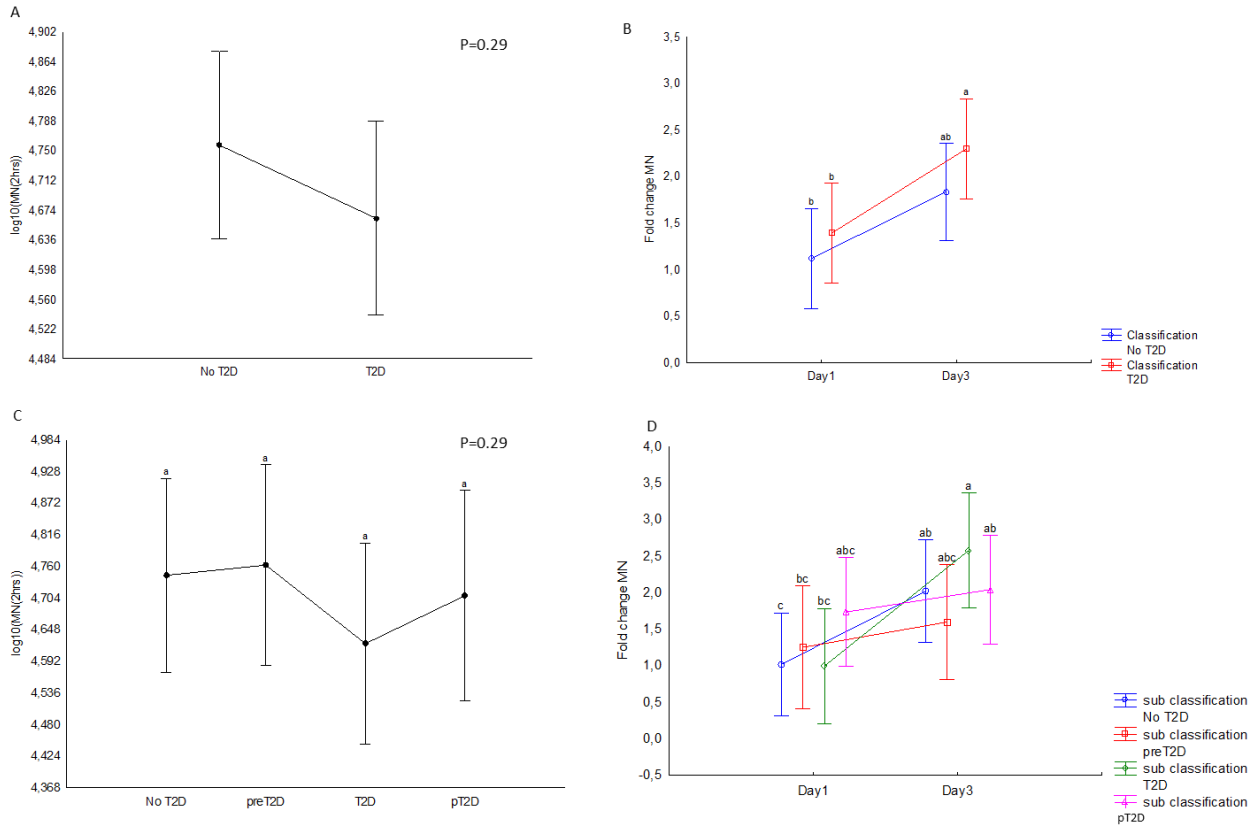

Figure 4.10 The influence of T2D on the containment of Mtb by MNs. MNs from LTBI-noT2D (HbA1c 5.7-6.4%, n=20) and LTBI-T2D (HbA1c ≥6.5%, n=20) (A and C) and MNs from LTBI-noT2D (HbA1c 4.0-5.69%, n=10), LTBI-preT2D (HbA1c 5.7-6.49%, n=10), LTBI-T2D (HbA1c 6.5-8%, n= 10) and LTBI-pT2D (HbA1c ≥8%, n=10) (B and D) were infected with Mtb for two hours. CFUs counts were determined for uptake at two hours and Mtb burden and control at D1 and D3. The CFU data was analysed using ANOVA with a Fisher LSD *post hoc* test. Data is represented as LS means with 95% CI. Letters a-c indicate statistical significance with the same letter are not statistically different from each other.

4.3.13 No association between FBG and HbA1c with Mtb uptake by PBMCs

There was poor Mtb association with PBMCs from T2D patients. Following that observation, we did a spearman correlation between the clinical characteristics associated with T2D. We found a negative correlation between BMI and Mtb uptake by PBMC (p=0.07, r=-0.29) of patients with LTBI and T2D, however this was only a trend. There was no correlation between FBG and Mtb uptake by MNs (p=0.14; r=0.25; Figure 4.11A) and PBMCs (p=0.14, r=0.25; Figure 4.11B). There was also no relationship between Mtb uptake by MNs (p=0.12, r=0.27; Figure 4.11C) and PBMCs (p=0.82, r=-0.04; Figure 4.11D) with HbA1c.

When the participants were further divided into the four groups according to HbA1c (LTBI-noT2D, LTBI-preT2D, LTBI-T2D and LTBI-pT2D), FBG positively correlated with Mtb uptake

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in PBMCs of LTBI-noT2D (p=0.03, r= 0.68) as well as in LTBI-T2D (p=0.01, r= 0.75; data not shown). There was no correlation between FBG and Mtb uptake by PBMC of LTBI-preT2D (p=0.25, r=0.40) and LTBI-pT2D (p=0.73, r=0.13; data not shown).

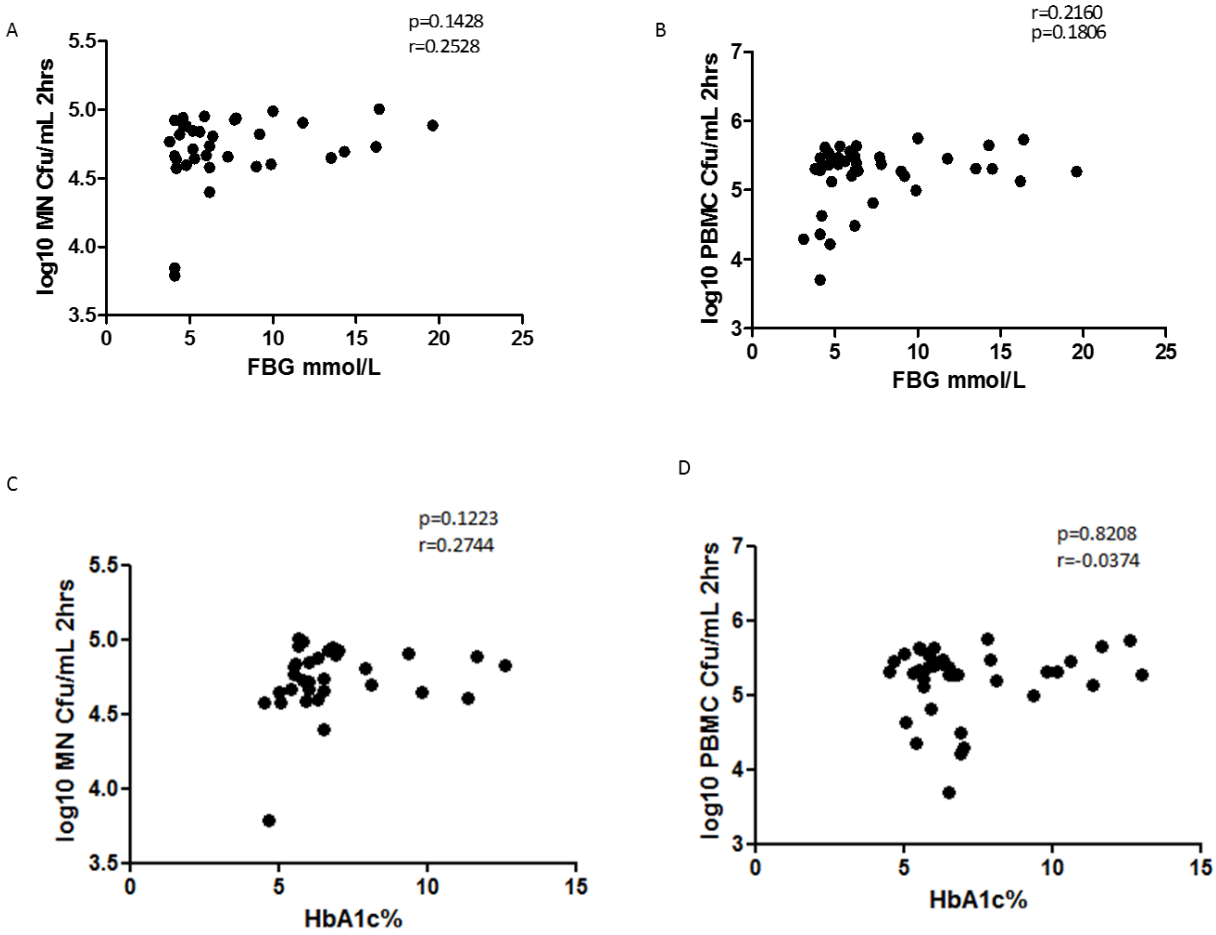

Figure 4.11 Correlation of glycaemic control with Mtb uptake. FBG was correlated to Mtb uptake by MNs (A) and PBMCs of (B). HbA1c was correlated to Mtb uptake by MNs (C) and PBMCs (D). The associations were determined using Spearman correlations.

4.3.14 Correlations between Mtb killing and cytokines

We observed that IL-6, IL-18 and IL-22 serum concentrations were high in T2D patients and investigated further whether there were associations between the cytokine concentrations and the Mtb uptake and killing. Firstly, we did correlations of cytokines and CFU data in all participants. IL-6 did not shown any significant correlation with Mtb uptake by MNs (p=0.19, r=-0.23; Figure 4.12A) or PBMCs (p=0.63, r=-0.08; Figure 4.12B) and the same was seen for IL-18 and IL-22. I-TAC showed a trend towards a positive correlation with Mtb uptake by PBMCs (p=0.06, r=0.30; data not shown) and IL-4 a trend towards a negative correlation with D1 CFU fold change in PBMCs (p=0.09, r=-0.27; data not shown). I-TAC was negatively correlated to D6 CFU fold change in PBMCs (p=0.01, r=-0.49).

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In the analysis of the four groups, we found that IL-6 was positively correlated with Mtb uptake by PBMCs in the LTBI-noT2D group (p=0.03, r=0.68; data not shown) while IL-22 showed a trend towards a negative correlation with Mtb uptake by PBMCs in the same group (p=0.06, r=0.71). IL-6 was negatively correlated to D1 CFU fold growth in PBMCs (p<0.01, r=-0.91). In addition, I-TAC revealed a trend towards a negative correlation with D6 CFU fold growth in PBMCs (p=0.05, r=-0.64).

In LTBI-preT2D, IL-6 was not significantly correlated to Mtb uptake by PBMCs (p=0.13, r=-0.52; data not shown). IL-4 was positively correlated to D3 CFU fold growth in MNs (p=0.03, r=0.59) and a similar association was found with IL-18 and D3 CFU fold change in MNs (p=0.04, r=0.76). A trend towards a positive correlation was seen between IL-8 and D3 CFU fold change in MNs (p=0.07, r=0.69). IL-4 (p=0.08, r=-0.66) and IL-8 (p=0.07, r=-0.71) revealed a trend towards a negative association with Mtb uptake by MNs.

IL-6 was positively correlated with Mtb uptake by PBMCs in LTBI-T2D patients (p=0.02, r=0.72; data not shown). Furthermore, IL-6 showed a trend towards negative correlation with D6 CFU fold change in PBMC (p=0.06, r=-0.61). IL-13 (p=0.04, r=-0.65) and I-TAC (p=0.02, r=-0.73) negatively correlated with fold change in PBMCs at D6. IL-18 on the other hand was trending towards a negative correlation with Mtb uptake by MNs (p=0.05, r=-0.74)

There was a negative correlation between IL-6 and Mtb uptake by PBMCs of LTBI-pT2D (p=0.01, r=-0.75; data not shown), similarly, OSM was also negatively correlated to Mtb uptake by PBMC (p=0.04, r=-0.72).

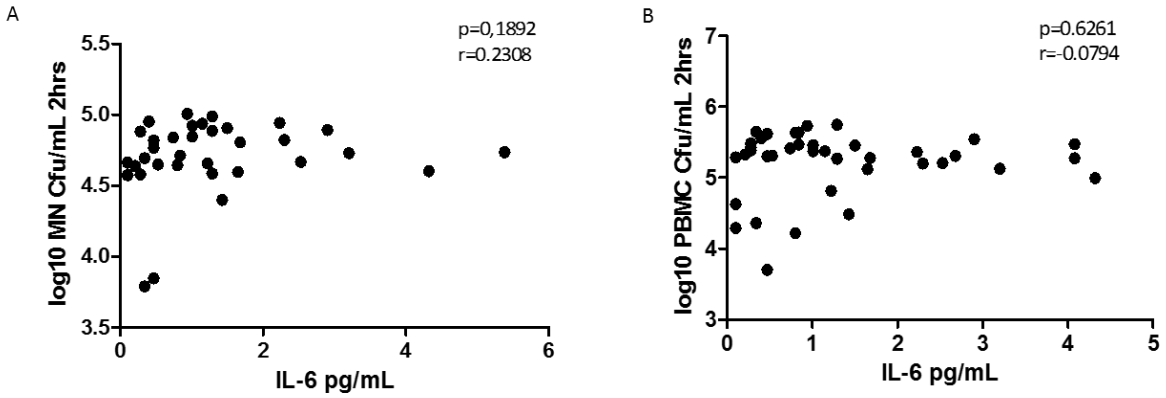

Figure 4.12 Correlation of serum cytokines with Mtb uptake by PBMCs. (A) IL-6 correlated to Mtb uptake by MNs (A) and PBMCs (B). The associations were determined using Spearman correlations.

4.3.15 Correlations between Mtb killing and measured catecholamines, cortisol, insulin and vitamin D

A correlation analysis was done to determine whether plasma hormone concentrations are associated with differences in CFU. This is relevant as PBMCs and MNs were cultured in 20% autologous plasma. There was no significant association between Mtb uptake by MNs

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(p=0.78, r=0.-0.05; Figure 4.13A) and PBMCs with vitamin D (p=0.66, r=-0.08; Figure 4.13B) and other measured hormones. There was a weak positively correlated between Vitamin D and D1 CFU fold growth in PBMCs (p=0.02, r=0.39).

When groups were subdivided according to HbA1c, vitamin D in LTBI-noT2D did not correlate with Mtb uptake by PBMC (p=0.50, r=0.29). A trend towards a positive correlation between cortisol and Mtb uptake by MNs (p=0.09, r= 0.58) was shown in the same group. Adrenaline measured in LTBI-noT2D also showed a trend to negatively correlate with D3 CFU fold change in MNs (p=0.08, r=-0.63).

Vitamin D concentrations from LTBI-preT2D correlated negatively with Mtb uptake by PBMCs (p=0.02, r=-0.73). There was a trend for adrenaline to positively correlate with Mtb uptake by PBMC (p=0.06, r=0.62). In addition, in LTBI-preT2D there was a negative correlation between cortisol and CFU fold change in PBMC at D1 (p=0.01, r=-0.77) and a trend for a positive correlation between adrenaline and D1 fold change in PBMCs (p=0.08, r=0.59).

Adrenaline measured in T2D patients was negatively correlated to Mtb uptake by PBMCs (p<0.01, r=-0.81). Vitamin D had a positive correlation with Mtb uptake by PBMC (p=0.04; r=0.72, Figure 4.13C). Serotonin showed a positive association with the CFU fold change from PBMCs at D1 (p=0.04; r= 0.62). Adrenaline was negatively correlated to Mtb uptake by MNs (p=0.02, r=-0.81) in T2D patients.

Furthermore, dopamine positively correlated with D1 fold change in MNs in pT2D patients, however this was not statistically significant (p=0.07, r=0.69).

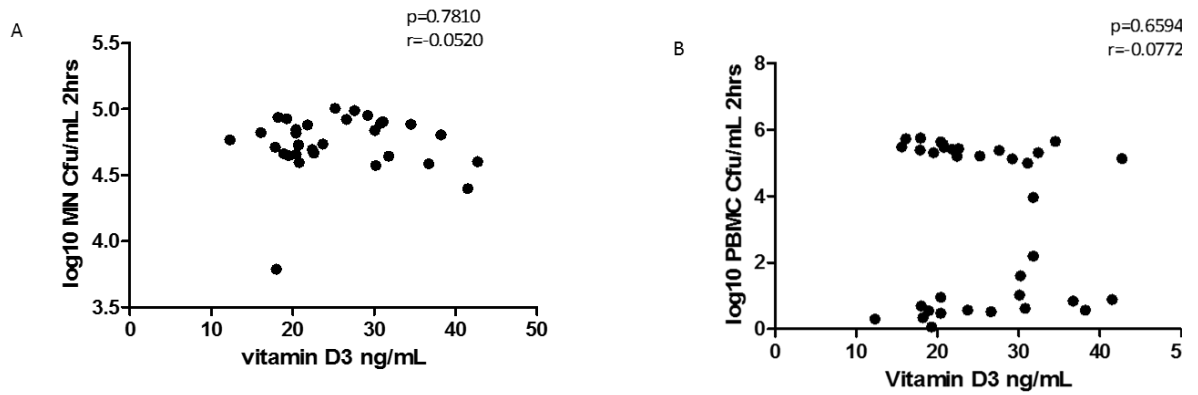

Figure 4.13 Correlation of plasma hormones with Mtb uptake by PBMCs. Vitamin D was correlated to Mtb uptake by MNs (A) and PBMCs (B). The associations were determined using Spearman correlations.

4.3.16 Correlations between Mtb killing and lipids

Most of the patients with T2D are characterised by dyslipidaemia and it is believed that this dyslipidaemia play a role in susceptibility to TB disease (Martens et al. 2008). We wanted to determine the association between lipids and Mtb uptake and killing. In all the participants, there was no association between any of the lipids and Mtb uptake by MNs (p=0.22, r=0.21;

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Figure 4.14A) or PBMCs (p=0.10, r=-0.26; Figure 4.14B), total CHL is shown as a representative of the hormone data. A trend for a positive correlation between LDL and D1 CFU fold change in PBMC.

When the participants were subdivided according to HbA1c, lipids of LTBI-noT2D did not correlate with CFUs.

In LTBI-preT2D there was a trend towards negative correlation between triglycerides and D1 CFU fold change in PBMCs (p=0.06, r=-0.61) of the same group.

Total CHL was negatively correlated with Mtb uptake by PBMCs in LTBI-T2D (p=0.02, r=-0.73; data not shown). There was a trend for a negative correlation between LDL and Mtb uptake by PBMCs (p=0.06, r=-0.62). In MN cultures, there was a negative correlation between total CHL and Mtb uptake in LTBI-T2D (p=0.05, r=-0.75). LDL showed a trend for a negative correlation with Mtb uptake by MNs (p=0.08, r=-0.67). Triglycerides were trending towards a negative correlation with D3 CFU fold change in MNs in the same group (p=0.05, r=-0.74).

In LTBI-pT2D, there is a trend towards a negative correlation between LDL and Mtb uptake by MNs (p=0.09, r=-0.71).

This shows that there are some interplay between the lipids in the plasma and the ability of cells to control Mtb. In our ALERT cohort, we found that LDL was associated with reduced Mtb growth (manuscript in preparation), which we did not find in the smaller subset of individuals in this study.

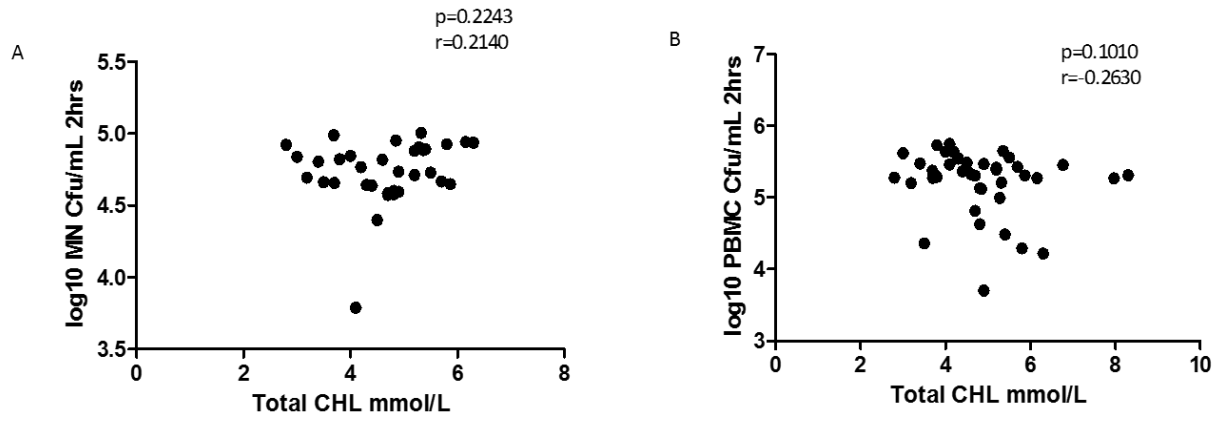

Figure 4.14 Correlation of lipids with Mtb uptake by PBMCs. Total CHL was correlated to Mtb uptake by MNs (A) and PBMCs (B). The associations were determined using Spearman correlations.

4.3.17 Correlations between Mtb killing and complete blood counts

We observed that there was poor association of Mtb with PBMC from T2D patients, therefore, we investigated whether correlations exist between the frequency and the absolute counts of the blood immune cells and Mtb killing. In all participants, there was no association between

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Mtb uptake by MNs (p=0.42, r=-0.15; Figure 4.14A) or PBMCs (p=0.84, r=0.03; Figure 4.14B) and white cell counts.

Whole blood cell counts from LTBI-noT2D showed a trend to positively correlate with Mtb uptake by PBMCs (p=0.08, r=-0.70; Figure 4.15A). Lymphocyte frequency was negatively correlated with Mtb uptake by PBMCs (p=0.03, r=-0.69). Still in LTBI-noT2D, the lymphocyte frequency also showed a trend towards a positive correlation to D1 fold change in PBMCs (p=0.07, r=0.60) and D6 fold change in PBMCs (p=0.09, r=0.57). The MN absolute count was also negatively correlated with D6 CFU fold change in PBMCs (p<0.01, r=-0.81).

In LTBI-preT2D, white blood cell counts had no correlation with Mtb uptake by PBMC (p=0.39, r=0.33; Figure 4.15B). MN frequency was positively correlated to D6 PBMC CFU fold growth (p=0.04, r=0.73). White blood cell counts (p=0.09, r=0.71) only showed a trend towards a positive correlation with Mtb uptake by MNs, additionally, white blood cell counts were inversely correlated to D3 MN CFU fold growth (p=0.03, r=-0.82).

In LTBI-T2D, the whole blood cell count was not correlated to Mtb uptake by PBMC (p=0.61, r=0.19). MN frequency had a trend towards negative correlation with Mtb uptake by PBMC (p=0.08, r=-0.57). MN frequency was negatively associated with Mtb uptake by MNs at two hours (p=0.04, r=-0.73). Lymphocyte frequency from T2D patients was negatively correlated to D1 MN CFU fold growth (p<0.01, r=-0.90).

In LTBI-pT2D, there was no association between white blood cell counts and Mtb uptake by PBMC (p=0.41, r=0.30). MN frequency from pT2D patients had a trend to a negative correlation with D1 PBMC fold growth (p=0.05, r=-0.63), further we showed a trend for a negative correlation between MN count and D6 PBMC fold growth (p=0.08, r=-0.59).

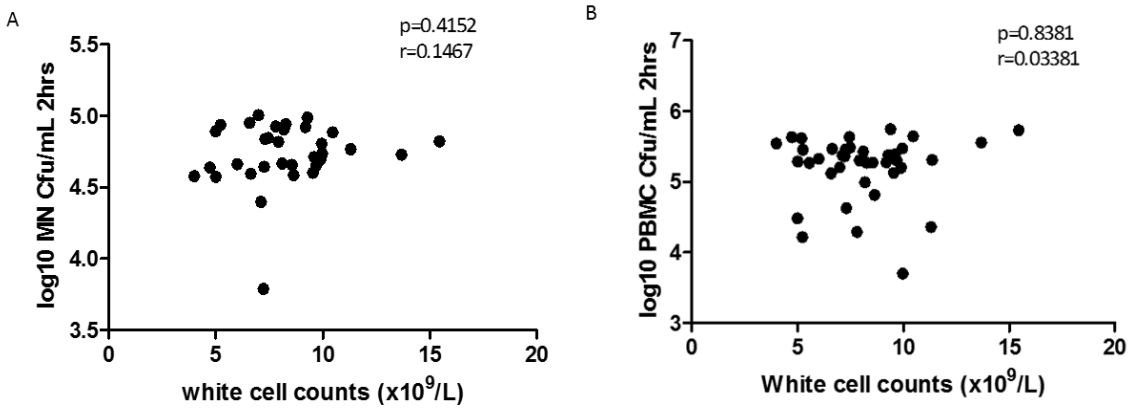

Figure 4.15 Correlation of differential blood counts with Mtb uptake. White blood cell counts correlation to Mtb uptake by MNs (A) and PBMCs (B). The associations were determined using Spearman correlations.

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4.4 Discussion

The chronic inflammation accompanying TB disease is associated with altered cellular immunity, as well as altered cytokine production (Kumar et al. 2014; Kumar et al. 2015; Pearson-stuttard et al. 2015; Kumar et al. 2016). Studies have been conducted in an attempt to understand the influence of T2D in the control of Mtb infection (Restrepo et al. 2008; Alim et al. 2017; Raposo-Garcia et al 2017; Boillat-Blanco et al. 2018). With this in mind, we studied the immune profile in patients with T2D residing in a high TB burden area in SA. Therefore, we investigated the influence of T2D on the immune response during Mtb infection of PBMCs and MNs isolated from LTBI individuals with and without T2D and further analysed the cytokines, hormones, lipids and differential blood cell counts in these participants.

TNF-α is an important cytokine for the immune response during Mtb infection (Shim, 2014). Individuals with LTBI receiving TNF-α antagonists are at high risk of developing TB disease (Tobin et al. 2011). In a study that investigated immunological responses to Mtb infection in T2D patients and healthy controls, the levels of TNF-α were higher in the T2D patients (Raposo-Garcia et al. 2017). TNF-α was however, not detected in the serum of our participants. TNF-α was reported to be low in Mtb antigen stimulated whole blood and Mtb infected PBMCs of T2D patients (Kumar et al. 2015; Alim et al. 2017). The fact that TNF-α was below the detection limit in our study participants could be due to the nature of samples we used, which was serum while other studies measured this cytokine in either Mtb stimulated or infected samples. IFN-γ is also a crucial cytokine in anti-mycobacterial immunity (Tascon et al. 1998). IFN-γ plays a central role in Th1 responses required to clear Mtb infection. Mice incapable of producing IFN-γ presented with a high Mtb burden in the spleen and lungs after intraperitoneal Mtb infection (Tascon et al. 1998). In a recent study investigating the immunological responses to Mtb infection in T2D patients and healthy controls, IFN-γ could not be detected in T2D patients (Raposo-Garcia et al. 2017). In our study we also saw similar results where the concentration of IFN-γ was undetectable in plasma of LTBI-T2D.

IL-23 along with IL-1β and IL-6 are essential for Th17 differentiation (Annunziato et al. 2012) and inducing IL-22 production (Rutz et al. 2013). Low concentration of IL-22 in LTBI-T2D patients in our study could be associated with the low levels of IL-23 and IL-1β cytokines especially since they were undetected, with the exception of IL-6. Dhiman et al. showed that high IL-22 is associated with protection in infectious diseases (Dhiman et al. 2009). Contrary to our finding, Kumar et al. reported higher IL-22 in the serum of LTBI-T2D patients compared to LTBI-noT2D (Kumar et al. 2015). This difference could be attributable to ethnicity since Kumar and colleagues investigated this in Chennai, India, and it has been shown that patients with T2D have metabolic differences depending on where they are from (Restrepo et al. 2018), which means that there might be other factors like cytokine which differ. Together with IL-17,

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IL-22 promotes lung repair, the release of pro-inflammatory chemokines and cytokines, and antimicrobial proteins (Rutz et al. 2013; Ronacher et al. 2018).

Despite its protective role and wound healing properties, excessive production of IL-22 can be harmful and induce necrosis. IL-22 further reduces blood glucose levels, restores insulin sensitivity and modulate lipid metabolism in mice (Hasnain et al. 2014). In humans, Kumar et al. reported low IL-22 in PPD stimulated whole blood and in plasma of TB-T2D patients compared to TB patients (Kumar et al. 2013; Kumar et al. 2015) which could be associated with the disease progression of T2D in TB disease (Treerat et al. 2017) or can favour Mtb control in TB. IL-22 concentrations in our TB-T2D patients was not significantly different compared to TB-THG but was higher compared to TB patients. This was contrary to what Kumar et al. found in their study where IL-22 was significantly lower in TB-T2D compared to TB patients (Kumar et al. 2015). Higher IL-22 concentration in our study may be a way of the immune system to promote metabolic balance and control of TB disease. However, the differential expression of IL-22 in our patients and varying results in previous studies supports the importance of IL-22 in the regulation of the immune response, and warrants further investigation to understand the exact role of IL-22.

IL-18 production is low in Mtb antigen stimulated whole blood of LTBI-T2D compared to LTBI without T2D (Kumar et al. 2014). We however found higher IL-18 in the LTBI-pT2D compared to LTBI only. IL-18 plays a critical role in inducing Th1 responses during Mtb infection (Schneider et al. 2010; Ponnana et al. 2017), without IL-18, the Th1 responses are decreased and mycobacterial replication increases (Schneider et al. 2010). In our study, we expected lower IL-18 expression in LTBI-T2D compared to LTBI since T2D is associated with increased susceptibility to TB progression. However, due to chronic inflammation in T2D, increased IL-18 may be due to dysregulated immune responses associated with T2D. In our mycobacterial infection assay, we found a negative correlation between IL-18 and Mtb uptake by MNs in LTBI-T2D and positively correlated to Mtb burden in MNs at D3. Our finding therefore show that IL-18 was associated with poor association of Mtb with MNs in T2D patients and with poor control of Mtb by MNs.

IL-6 is a pro-inflammatory cytokine and its upregulation is associated with inflammatory diseases such as TB (Martinez et al. 2013) and T2D. IL-6 is required for the rapid expression of an initial IFN-γ response during Mtb infection (Saunders et al. 2000). This is because IL-6 knock out mice showed increased bacterial growth in the lung within 14 days of Mtb infection compared to wild type mice (Saunders et al. 2000). In TB-T2D, IL-6 plasma concentrations are high compared to TB (Kumar et al. 2013). We measured IL-6 in LTBI-T2D, and we found high IL-6 compared to LTBI. IL-6 has the ability to impair insulin signalling in insulin-sensitive tissues (Nikolajczyk et al. 2012). We believe that higher IL-6 in T2D is due to chronic low grade

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inflammation and could be associated with insulin resistance since it is one of the characteristics of T2D patients. Lower IL-6 production by macrophages from diabetic mice infected with *Mycobacterium fortuitum* contributed to increased bacterial growth in the infected macrophages which suggests that IL-6 is required for better control of bacteria (Abdul Alim et al. 2017). Despite the fact that IL-6 is a pro-inflammatory cytokine, its high expression in our study was associated with poor Mtb uptake by PBMCs from T2D patients. As previously mentioned in our findings, T2D patients had lower Mtb uptake by PBMCs compared to noT2D, with that in mind, we also think that IL-6 may promote immune suppression in T2D patients (Zhang et al. 2014).

Vitamin D inhibits intracellular growth of Mtb by interfering with the accumulation of infection induced lipid droplets (Salamon et al. 2014). Contrarily, vitamin D can suppress pro-inflammatory cytokines and induce anti-inflammatory cytokine responses (Penna and Adorini 2000; Unger et al. 2009). Bhatt et al. investigated whether administration of vitamin D could affect immunity against Mtb in mice and found that vitamin D affected granuloma formation and failed to contain the bacteria (Bhatt et al. 2016). In our study, there was a trend that T2D patients had higher levels of vitamin D compared to noT2D. Banerjee et al. however found that vitamin D concentration were lower in the serum of T2D patients compared to noT2D (Banerjee et al. 2017). We further found that vitamin D was positively correlated to Mtb uptake in PBMCs. This is consistent with previous reports that vitamin D significantly enhances the phagocytic potential of macrophages isolated from healthy individuals (Chandra et al. 2004). In a study where TB patients were given vitamin D while on intensive phase of TB treatment, the bacterial load was reduced *in vitro* as measured by reduced time to sputum conversion (Martineau et al. 2014).

T2D is characterized by hyperglycaemia due to insulin resistance and also associated with severe dyslipidaemia (Taskinen and Boren, 2015). Dyslipidaemia is characterized by high levels of triglycerides, and/or cholesterol as well as low HDL (Vrieling et al. 2018). In our study LTBI-pT2D patients had higher triglycerides and total CHL compared to noT2D, preT2D and T2D patients. LDL was also higher in LTBI-pT2D compared to LTBI-noT2D, followed by higher HDL in LTBI-noT2D compared to LTBI-T2D. Mtb has been shown to depend on host-derived lipids for replication and survival (Lee et al. 2013; Tsai et al. 2017; Walpole et al. 2018). Several studies have identified high cholesterol as a risk factor for TB disease (Martens et al. 2008; Soh et al. 2016) and reducing cholesterol levels using statins were beneficial in reducing the risk of TB disease (Lai et al. 2016; Su et al. 2017). In our study, total CHL in T2D patients was negatively correlated to association of Mtb with PBMCs, which shows the interplay between lipids and Mtb uptake. A potential therapy targeting cholesterol catabolism can effectively lower intracellular growth of Mtb (Tsai et al. 2017) and more studies should focus on

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investigating the use of CHL lowering drugs in conjunction with TB treatment to prevent TB disease especially in LTBI-T2D.

The leukocytes (white blood cells, neutrophils, lymphocytes, MNs and eosinophils) counts are elevated in patients with T2D (Shim et al. 2006; Nakhjavani et al. 2014). Higher white blood cell and lymphocyte counts are characteristics of T2D development in obese patients (Vatcheva et al. 2014). In support of that, we found higher white blood cell counts in uncontrolled T2D (pT2D) patients compared to noT2D. Our study found lymphocyte counts significantly higher in the pT2D patients compared to noT2D, preT2D and T2D. This was similar to other reports where pT2D patients had elevated lymphocyte counts (Ali et al. 2018). T2D exerts adverse effect on neutrophils and MNs functions (Geerlings and Hoepelman 1999; Raposo-Garcia et a. 2017). Although higher in T2D patients, no significant difference was observed in neutrophil counts in our study participants, however, Ali et al. found significantly elevated neutrophil counts in T2D compared to noT2D (Ali et al. 2018). The negative correlation observed in our study for neutrophils and MNs with Mtb uptake in T2D patients has been shown before (Lecube et al. 2011; Gomez et al. 2013; Raposo-Garcia et al. 2017).

It is well established that T2D is associated with altered MN and neutrophil functions (Hatanaka et al. 2006; Alba-Loureiro et al. 2007; Restrepo et al 2014). Despite the evidence that MNs from TB naïve T2D patients are altered (reduced phagocytosis) (Restrepo et al. 2014; Raposo-Garcia et al. 2017), we cannot say for sure that these alterations are the only factors contributing to the altered immune responses observed in T2D patients, because in our study, the MNs did not show any difference in *H37Rv* Mtb uptake between T2D and noT2D participants. Additionally, in a recent study, the lipid profile was shown to vary in T2D patients from different ethnic groups, and we believe that this variation could also affect the function of the phagocytic cells (Restrepo et al. 2018). The killing ability of MNs from both noT2D and T2D was not different, as shown by a significant increase in CFU at D3 in each group. Our results supported previous studies where no difference in Mtb killing by MNs derived macrophages, and blood were observed (Reyes-Ruvalcaba et al. 2008; Raposo-Garcia et al. 2017).

Our results adds to the small body of knowledge that is available on the immune profile, including cytokine production, hormone production and the ability to contain bacteria by immune cells from individuals with LTBI with and without T2D. We have shown that there is a need for more studies to define the immune profile of these individuals, to help us learn and understand the immune mechanisms during Mtb infection and influence of plasma soluble proteins and lipids in Mtb phagocytosis and killing assays.

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CHAPTER 5

Investigation of MAIT cells in TB patients with and without T2D

5.1 Introduction

MAIT cells predominantly occur in the gut where their accumulation is dependent on MR1 expressing B cells and commensal flora (Martin et al. 2009). They also occur in the blood where 1-10% of T cells are MAIT cells (Dusseaux et al. 2011). The frequency of MAIT cells in bacterial and viral infections, as well as in metabolic and autoimmune diseases vary (Billerbeck et al. 2010; Magalhaes et al. 2015; (Loh et al. 2016; Petersone and Walker, 2017), implying that they might play an important role in the regulation of immune responses in these diseases. It is said that low frequencies of MAIT cells in the blood during Mtb infection is due to their migration to the site of infection (Gold et al. 2010). Wong et al. also demonstrated this after measuring MAIT cell frequencies in the Broncho alveolar lavage fluid (BALF) and blood of TB patients and TB-HIV co-infected patients (Wong et al. 2012). Higher frequencies of MAIT cells were found in the BALF than in the periphery. The addition of MAIT cells to BCG-infected macrophages resulted the increased killing of the bacteria demonstrating the protective role MAIT cells play during Mtb infection (Chua et al. 2012). MAIT cells not only assist in the control of bacterial infection, but also in the detection of bacterially infected cells (Gold et al. 2010; Le Bourhis et al, 2010).

In the context of T2D, it has been shown that patients with T2D have a reduced peripheral MAIT cell frequency when compared to HCs (Magalhaes et al. 2015). This is in part due to the alterations in the gut microbiota in T2D patients (Vaarala and Atkinson, 2008.), which affects the riboflavin pathway which is essential in MAIT cell activation (Treiner et al. 2003). The exact role of MAIT cells in metabolic diseases is, however, unclear. The aim of this study, therefore, was to determine the frequency of MAIT cells in the South African Coloured population residing in a high TB burden area in Cape Town, South Africa. The frequency of MAIT cells were determined in the following groups; HCs, T2D, TB, TB-THG and TB-T2D using flow cytometry. In addition, MAIT cell frequencies were monitored before, during and after TB treatment in TB subgroups.

5.2 Study participants and methodology

Cryopreserved PBMCs from 64 participants (TANDEM); were retrieved, thawed and stained with a MAIT cell flow cytometry antibody panel. The flow cytometry panel consisted of the following anti-human monoclonal antibodies; CD3, CD4, CD8, CD161, CD26 and TCR Vα-7.2 as well as a live/dead (Zombie aqua) stain. PBMCs from HCs, T2D, TB, TB-THG, and TB-T2D patients were stained and the frequency of MAIT cells compared at baseline (BL). In addition, PBMCs from TB, TB-THG and TB-T2D patients were stained at month 2 (M2) and month 6 (M6) to monitor the frequency of MAIT cells during and after TB treatment. The

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PBMCs were stained for one hour at 4°C in 2% FACs buffer. Cells were acquired using a 4 laser, 8 colour FACS Canto II flow cytometry instrument (Becton Dickson). The results were analysed using FlowJo v10.1 (TreeStar, Ashland, OR). At first, lymphocytes were selected using SSC-A and FSC-H, doublets were excluded using FSC-A and FSC-H and live cells selected using SSC-A and the live/dead stain (Figure 5.1). $CD8^{+}$TCR $V\alpha7.2^{+}$ T cells were selected and from this population $CD26^{++}$ $CD161^{++}$ cells were selected. Flow data was analysed using ANOVA and LSD Fishers *post hoc* test were used to determine differences between the groups. Differences between clinical information was determined in GraphPad prism version 6 using the Kruskal Wallis with Dunns *post hoc* to determine differences between the groups. A p-value < 0.05 was considered significantly different. Spearman correlation was performed to determine correlations between MAIT cells and blood glucose levels.

5.3 Results

5.3.1 Study participants and clinical parameters

Among the 64 participants that were selected for this study, 17 were TB patients, 9 were TB-THG, 13 were TB-T2D and 13 were T2D only patients. Twelve HCs were also included. Patients with T2D were using metformin or metformin in combination with insulin and other T2D medication (Table 5.1). There were no significant differences in the ages of the different patient groups (Table 5.1). The BMI at BL was significantly higher in T2D and TB-T2D patients compared to the HCs, TB and TB-THG patients (Table 5.1). Both at M2 and M6, there was significantly higher BMI in the TB-T2D group compared to TB and TB-THG. RPG was significantly higher in the TB-T2D patients compared to T2D and HCs. HbA1c was significantly higher in the TB-T2D and T2D patients compared to TB patients, whereas HbA1c in T2D was also significantly higher than the HCs. At M6 however, HbA1c in the TB-T2D patients was higher compared to both TB and TB-THG (Figure 5.1).

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Table 5.1. Demographic and clinical information of all study participants.

Group	HC(12)	T2D(13)	TB(17)	TB-THG(9)	TB-T2D(13)	P value
LTBI positive (%)	10 (83%)	11 (85%)	-	-	-	
T2D medication						
Metformin	-	10	-	1	11	
Insulin	-	10	-	5	6	
Other medication	-	2	-	1	4	
Known diabetic status	-	-	-	-	-	
Sociodemographic						
Age, median (IQR)	40.5(19)[a]	44(19)[a]	47(14)[a]	48(16.5)[a]	46(11)[a]	0.6764
Sex, number (%)						
Female	6(50)	6(46)	7(41)	2(22)	11(85)	
Male	6(50)	7(54)	10(59)	7(78)	2(15)	
BMI, median (IQR)						
Baseline	23.4(10.2)[ab]	26.5(8.6)[a]	18.6(4.8)[cb]	18.3(0.8)[cb]	26.0(9.8)[a]	**<0.0001**
Month 2	-	-	19.4(4.0)[a]	19.5(2.2)[a]	24.9(6.5)[b]	**0.0005**
Month 6	-	-	20.2(4.4)[a]	19.3(1.7)[a]	26.6(9.4)[b]	**0.0116**
Clinical information						
RPG(mmol/L)						
Baseline[#]	5.1(1.2)[a]	15(6.3)[b]	5.6(2.1)[ac]	6.6(1.9)[ab]	18.4(4.1)[bc]	**<0.0001**
HbA1c (%)						
Baseline[#]	5.3(0.58)[ac]	10.3(4.71)[b]	5.3(0.38)[a]	5.6(0.65)[ab]	12.6(0.4)[bc]	**<0.0001**
Month 6	-	-	5.6(0.6)[a]	5.65(0.7)[a]	9.9(6)[b]	**<0.0001**

#- 2 participants in the TB-T2D group

5.3.2 TB-T2D have significantly low $CD3^+CD8^+$TCR $V\alpha7.2^+$ $CD26^{++}$ $CD161^{++}$ MAIT cells at baseline

MAIT cells were defined as $CD3^+$ $CD8^+$TCR $V\alpha7.2^+$ $CD26^{++}CD161^{++}$ and reported as a percentage of $CD8^+$ T cells (Figure 5.1). HCs had significantly higher MAIT cell frequency than TB-T2D at baseline (p=0.02). TB-T2D patients also had significantly lower MAIT cell frequency compared to TB-THG at BL (p=0.02; Figure 5.2 A, B and C). TB-T2D had lower MAIT cell frequencies compared to TB-THG at M2 (p=0.01; Figure 5.2 A and B). There was no significant difference in MAIT cell frequency between TB-T2D and TB at M2 (p=0.64). There was a trend in MAIT cell frequency at M2 between TB-THG and TB (p=0.08; Figure 5.2 A and B). There was no observed significant difference in MAIT cell frequency at M6 in all groups, despite a trend between TB and TB-THG, where TB-THG had higher MAIT cells (p=0.06; Figure 5.2 A-C).

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5.3.3 CD3^{+}CD8^{+}TCR Vα7.2^{+} CD26^{++} CD161^{++} MAIT cell frequency increases at month 2 of TB treatment

We further investigated whether TB treatment will have an effect on the frequency of CD8^{+}TCR Vα7.2^{+} CD26^{++} CD161^{++} MAIT cells in the three TB patient groups. The frequency of MAIT cells in TB patients did not change during TB treatment. There was a trend towards increase in MAIT cell frequency from BL to M2 (p=0.05) and a significant decrease from M2 to M6 (p=0.01) in the TB-THG group (Figure 5.2A and B). In the TB-T2D group there was a significant increase in the frequency of MAIT cells from BL to M2 (p=0.003), however, MAIT cell frequency from M2 to M6, was not significantly different (p=0.15) (Figure 5.2A and B). MAIT cell frequencies in this group was significantly lower at BL when compared to the end of treatment (p<0.0001; Figure 5.2A and B).These results show that TB-THG patients have varying MAIT cell frequency compared to TB and TB-T2D. The different treatment responses observed in the frequency of CD8^{+}TCR Vα7.2^{+} CD26^{++} CD161^{++} cells in these two group suggest that the TB-THG and TB-T2D are more different from one another than previously thought.

5.3.4 The frequency of CD3^{+}CD8^{+}TCR Vα7.2^{+} CD26^{++} CD161^{++} MAIT cells of TB-T2D patients at month 6 is comparable to T2D

We determined whether the frequency of MAIT cells in TB patients at M6, recovers to the levels seen in HCs. The frequency of CD3^{+}CD8^{+}TCR Vα7.2^{+} CD26^{++} CD161^{++} MAIT cells in the TB-T2D, which was lower at BL, recovered at M6 compared to frequencies similar to HCs (at BL) and all the other groups, Figure 5.2 A, C and D.

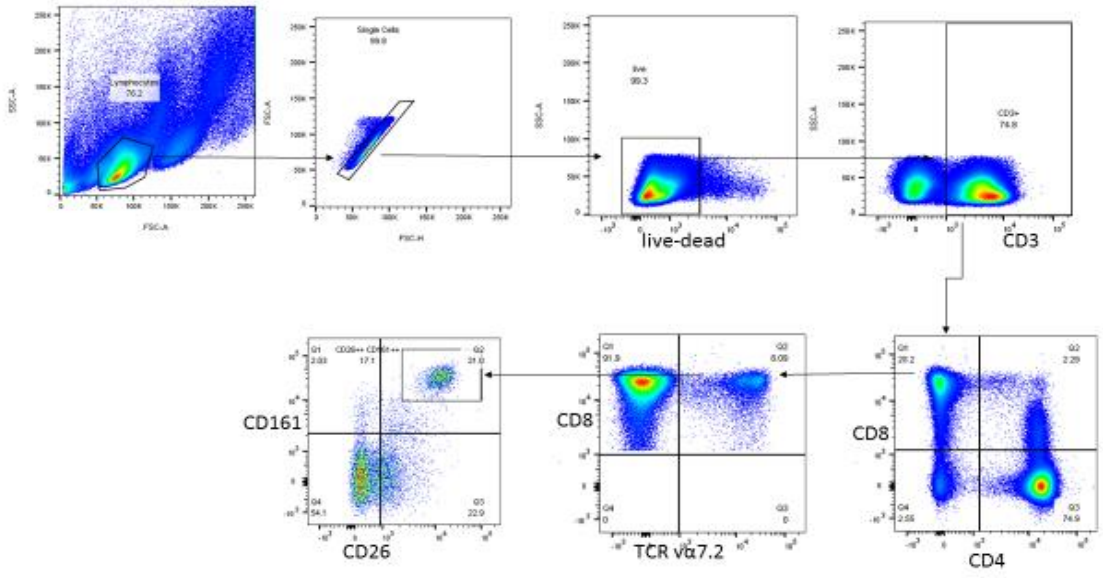

Figure 5.1. Characterization of circulating peripheral MAIT cells (CD3^{+}CD8^{+}TCR Vα7.2^{+} CD26^{++} CD161^{++}). PBMCs from HC, T2D, TB, TB-THG and TB-T2D were thawed and stained using anti-human antibodies for CD3, CD4, CD8, CD26, CD161 and TCR Vα7.2 for MAIT cells in 64 participants. All events were acquired using a 4

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laser, 8 colour FACS Canto II flow cytometry and analysed on flowJo v10.1. We gated on lymphocytes using SSC-A and FSC-H, doublets were excluded using FSC-A and FSC-H. Live cells were selected using SSC-A and the live/dead stain. $CD8^{+}$TCR $V\alpha7.2^{+}$ were selected from CD3 T cells, from this population $CD161^{++}CD26^{++}$ cells were selected.

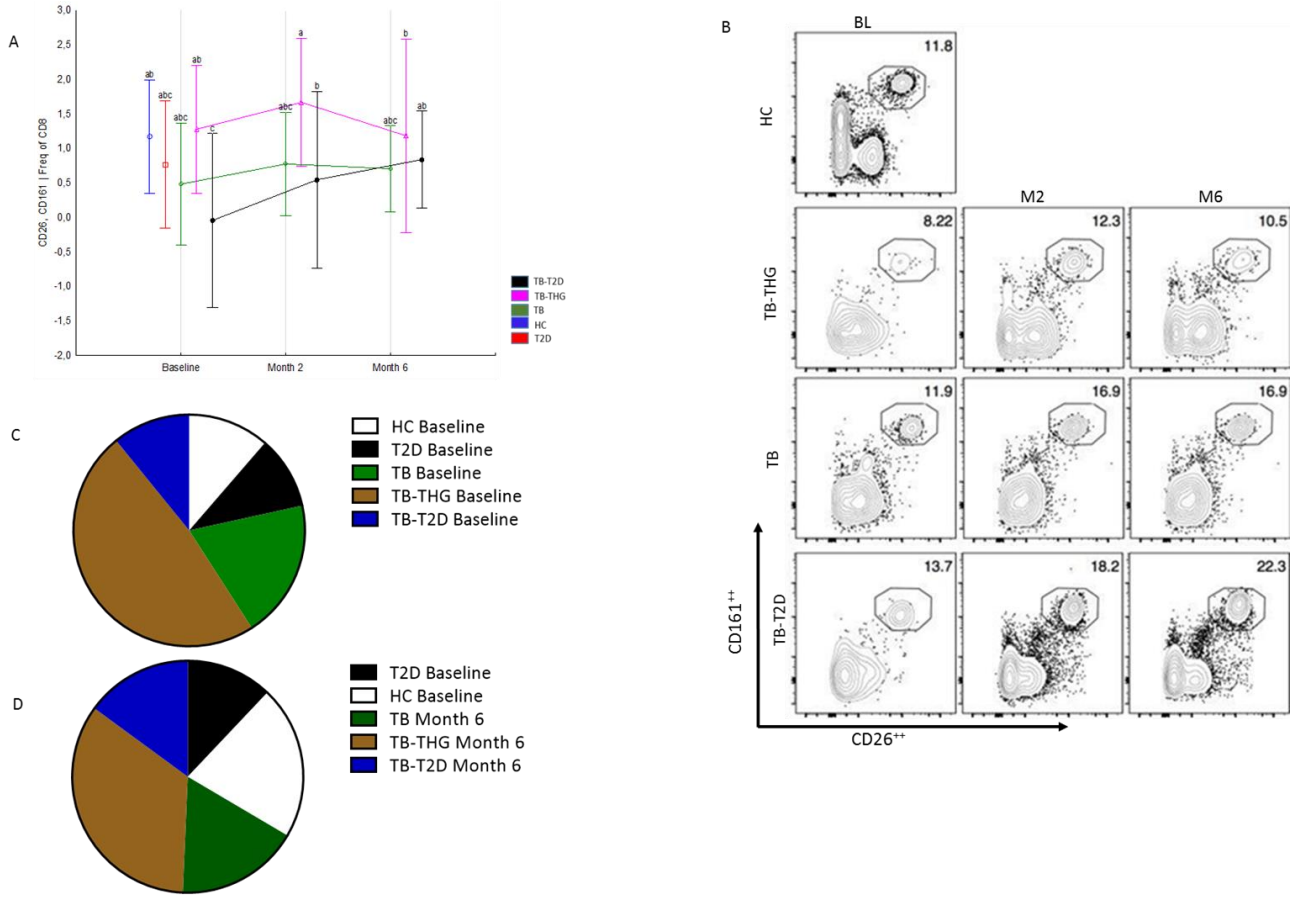

Figure 5.2: $CD8^{+}$TCR $V\alpha7.2^{+}$ $CD26^{++}$ $CD161^{++}$ MAIT cell frequencies. $CD8^{+}$TCR $V\alpha7.2^{+}$ $CD26^{++}$ $CD161^{++}$ MAIT cell frequencies from 64 participants (HCs n=13, TB n=17, T2D n=12, TB-THG n=9, TB-T2D n=13) were determined in PBMCs. Figure A and B illustrate the changes in MAIT cell population during TB treatment. Figure C and D show pie charts displaying the MAIT cell frequencies from T2D and HCs as well as TB, TB-THG and TB-T2D at BL and M6, respectively, ANOVA with a LSD *post hoc* test was used to determine differences in the frequencies of MAIT cells between the groups, the pie chart was drawn in GraphPad prism version 6. And a p-value <0.05 was considered significant. BL baseline, M2 month2, M6 month6.

5.3.5 Association of T2D parameters with $CD8^{+}$TCR $V\alpha7.2^{+}$ $CD26^{++}$ $CD161^{++}$ MAIT cells

HbA1c and RBG were measured in the study groups and was correlated with the MAIT cell frequencies. There was no correlation between MAIT cells and blood glucose levels (Figure 5.3 A and B).

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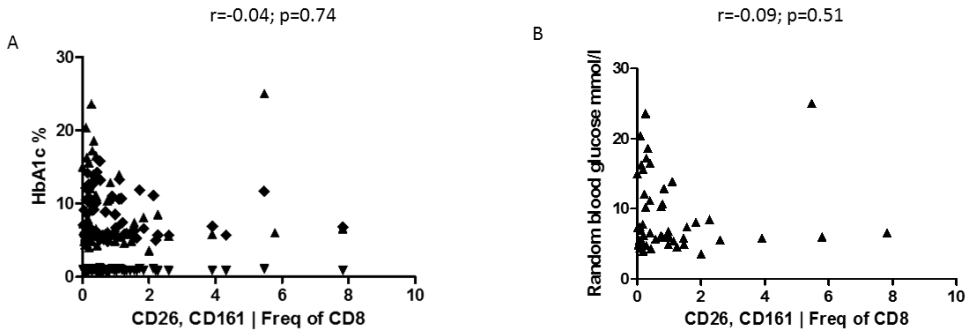

Figure 5.3 Correlation plots of MAIT cells with glycaemic parameters. There was no significant correlation between (A) MAIT cell frequencies and HbA1c (r=-0.04; p=0.74) or (B) MAIT cell frequencies and RBG (r=-0.09; p=0.51) using the Spearman ranks correlation test in Graphpad Prism version 6.

5.4 Discussion

MAIT cell frequencies range from 1-10 % of T cells in peripheral blood of healthy people (Kurioka et al. 2016). In this study an average of 1.14 % of MAIT cells ($CD8^{+}$TCR $V\alpha7.2^{+}$ $CD26^{++}$ $CD161^{++}$) were found in cryopreserved PBMCs. Such low frequencies have previously been reported from PBMCs. Paquin-Proulx et al. reported 1.07% in thawed PBMCs of HCs (Paquin-proulx et al. 2018). Lee et al. reported different MAIT cell frequencies in blood of HCs; 1.17%, 2.12% and 3.99 % according to the age of the study participants (Lee et al. 2014). The highest frequency was observed in the younger age group (21-40 years), followed by middle age (41-60 years) and elderly (61-92 years) (Lee et al. 2014). Since the depletion in MAIT cell frequencies is negatively associated with age (van der Geest et al. 2018), our study participants were matched by age. Therefore, in our study the association between MAIT cells and age was not observed.

There were differences in MAIT cell frequencies in our study compared to other reports (Gold et al. 2010; Le Bourhis et al. 2011; Jiang et al. 2014; Sakala et al. 2015). The reason could be attributable to the various phenotypes used to define the MAIT cell population. In this study we used the following phenotype $CD3^{+}CD8^{+}$TCR $V\alpha7.2^{+}$ $CD26^{++}$ $CD161^{++}$ (shared with us by David Lewinsohn). Many gating strategies have been used to describe circulating MAIT cell population. Eberhard et al. used $CD3^{+}CD4^{-}CD161^{+}$TRC7.2^{+}, Magalhaes et al. used $CD3^{+}CD4^{-}$ $CD161^{++}$TCR $V\alpha7.2^{+}$, and Wong et al. used $CD3^{+}CD8^{+}CD161^{++}$CCR6 and $CD3^{+}CD8^{+}CD161^{++}$CCR5 (Wong et al. 2013; Eberhard et al. 2014; Magalhaes et al. 2015). The use of the different markers in varying combinations to describe MAIT cells could be the reason for large variations in the findings from different reports.

MAIT cells play a key role in protection against several pathogens, including Mtb (Gold et al. 2010). Previous studies show that MAIT cell frequencies in the periphery decrease during TB and other bacterial infections compared to HCs (Gold et al. 2010; Wong et al. 2013; Grimaldi

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et al. 2014; Sharma et al. 2015). Contrarily, people with LTBI were shown to have higher MAIT cell frequencies compared to TB patients or uninfected controls. In addition to that, the authors also found no difference in MAIT cells in TB patients compared to uninfected controls (Paquin-Proulx et al. 2018). Our study supports the latter results since the reduction of MAIT cells in our TB patients were lower than the HCs who were all LTBI positive. In our data set, however, this difference did not reach statistical significance.

In a study investigating MAIT cells in obese and T2D patients before and after bariatric surgery, the MAIT cell frequency increased in the blood of patients who did not initially show any MAIT cells before surgery (Magalhaes et al. 2015). However, when MAIT cells were not in the blood, they were detected in adipose tissue and it was hypothesized that MAIT cells migrate to the site of inflammation (Magalhaes et al. 2015). What we found was in agreement with this since in uninfected T2D patients, the MAIT cells were lower than that of HCs, however not significant. The difference became apparent when MAIT cells were lower in TB-T2D patients. This result may be explained by the migration of MAIT cells to the adipose tissue (Magalhaes et al. 2015) or to the lung (Gold et al. 2010) as previously showed. There are no other published results on MAIT cells in TB-T2D co-morbidity.

TB is associated with increased insulin resistance and stress-induced hyperglycaemia or TB-THG, which may lead to over diagnosis of T2D during the early stages of TB disease (Boillat-Blanco et al. 2016). There was a trend towards higher frequency of MAIT cells in TB-THG compared to TB at M2. In addition, there was a significantly higher MAIT cell frequency in TB-THG compared to TB-T2D at M2 confirming that this subgroup of TB patients is unique from TB and TB-T2D. It could be that the high MAIT cell frequency in TB-THG patients is associated with protection from acquiring T2D in these patients. This would however, require a longitudinal study to determine if TB-THG patients develop T2D and if MAIT cell frequency plays a role.

TB patients, after TB treatment (M6), had a lower frequency of MAIT cells to that of the HCs (although not significant). It is possible that the residual infection, at M6, might still result in the recruitment of MAIT cells to the lung. In line with this finding, Paquin-proulx et al. reported that MAIT cells in individuals with LTBI was significantly higher compared to TB patients and speculated that high MAIT cells may be associated with Mtb control and protection from TB disease progression (Paquin-proulx et al. 2018). TB-T2D patients at M6 of TB treatment however had comparable frequency of MAIT cells to that in T2D patients. This is possible due to the clearance of Mtb and cause MAIT cells to redistribute to the periphery.

We investigated whether TB treatment will have an impact on the frequency of MAIT cells in TB patients and they did not change significantly during the six months of treatment. This is

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different from what was previously reported by Sharma et al. who showed that MAIT cells are restored in the blood of TB patients undergoing TB treatment (Sharma et al. 2015). It has been shown that the lack of MAIT cells in the blood during TB is due to redistribution to the lung (Gold et al. 2010; Wong et al. 2013). All the TB patients included in our study converted to sputum negative for Mtb at M2. We believe that due to the decrease in the bacterial load in the lung, some of the MAIT cells would be restored in the blood and some would be killed along with the bacteria at the lung. This could explain why there was no significant change in MAIT cells during the course of TB treatment. There are no other studies reporting MAIT cells treatment response during TB.

During the course of TB treatment, TB-THG patients showed a trend towards increased MAIT cell frequency from BL to M2. This increase would also be explained by the lower bacteria at the site of disease during treatment (Sharma et al. 2015). Interestingly, in TB-THG, the MAIT cell frequency significantly decreased from M2 to M6. This is different from what has been reported in the context of TB (Sharma et al. 2015). The fact that the patients are TB-THG and not TB only could be reason for this unexpected result. Gérart et al. reported that over activation of MAIT cells may result in activation induced cell death (Gérart et al. 2013), therefore, MAIT cells in the lung die due to apoptosis before they are restored to the blood.

The impact of TB treatment on MAIT cells was also investigated in TB-T2D patients. MAIT cells increased significantly from BL to M2 and are lower at BL compared to M6. Despite having T2D, the MAIT cell frequency in TB-T2D were restored in the periphery and this is in agreement with what was previously discussed by Sharma et al. in the context of TB patients (Sharma et al. 2015).

Chua et al. co-cultured MAIT cells with BCG-infected macrophages from TB patients and found fewer mycobacteria compared to macrophages without MAIT cells (Chua et al. 2012; Lepore et al. 2014). It is not known how T2D will affect the function of MAIT cells therefore more studies are required to understand their behaviour in different disease states, especially in a population where both TB and T2D poses a major problem. Moreover, MAIT cells play a role in setting a stage for establishing coordinated adaptive immune response to infection (Wong et al. 2016). Our patients with TB-T2D presented with reduced MAIT cells. Lower MAIT cell frequencies will most likely contribute to the dysregulated immune response against diseases (Kwon et al. 2015). MAIT cells play a protective role in TB, but their exact role in TB-THG or TB-T2D co-morbidity has not been characterized and requires further investigation especially in the blood and in BALF, which is a representative sample of the site of TB disease.

As expected we found higher MAIT cell frequencies in the HCs compared to TB-T2D patients at BL. Interestingly, in the TB-THG, the MAIT cells were higher at BL compared to the other

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groups (HC, TB and TB-T2D). Further characterisation of MAIT cells should be done in our patient group, looking at different phenotypes to fully understand their expression in different disease states. Seeing that there is limited information on MAIT cells in TB with and without T2D, we wanted to measure MAIT cell numbers in TB and TB-T2D patients and include LTBI-T2D as a control, however, in the end the LTBI-T2D group, as we did not have enough PBMCs available for this group.

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CHAPTER 6

6.1 Discussion

Hyperglycaemia during TB is associated with a high T2D risk and poor TB outcome (Moreira et al. 2018). Moreira et al. investigated the effect of hyperglycaemia in TB patients and found that a larger number of TB patients with hyperglycaemia exhibited adverse outcomes and a significantly higher mortality rate compared to euglycaemic patients (Moreira et al. 2018). T2D is a risk factor for TB and poor TB treatment outcome including treatment failure and relapse (Dooley et al. 2009) and for this reason the WHO, World Diabetes Foundation (WD) and the International Union Against TB and Lung Disease (IUATLD) recommend that all newly diagnosed TB patients are screened for T2D (Harries et al 2013). The TB-THG group is characterised by high HbA1c prior to TB treatment that decreases during the course of TB treatment (Tabarsi et al. 2014). It is therefore advised to monitor HbA1c of TB patients during TB treatment to confirm T2D status (WHO. 2018). Our study was able to identify a gene transcript signature that enables the differentiation between THG and T2D, which could be useful in preventing the unnecessary treatment of THG patients who were originally diagnosed as having T2D. Since HbA1c testing alone will not be able to distinguish patients with THG from T2D a gene signature could complement standard T2D screening of TB patients.

6.1.1 Gene expression signatures in active TB patients with and without T2D

Transcriptional analysis of whole blood and Mtb antigen stimulated whole blood has become an important tool to study the expression of immune system related genes and shows promise for identifying possible biomarkers for TB diagnosis, progression to active TB disease and TB treatment responses (Cliff et al. 2013; Zak et al. 2016; Thompson et al. 2017). Despite the usefulness of transcriptomic studies in addressing immune related responses, the exploration of transcriptional analyses in the context of TB-T2D co-morbidities is scarce (Prada-medina et al. 2017). Prada-Medina et al. showed that previously identified TB blood gene transcripts specific for diagnosing TB disease may not be affected by the presence of T2D (Prada-Medina et al. 2017). More studies in populations where TB-T2D co-morbidity is a burden would be of great significance for the identification of new molecular markers specific to T2D in TB patients to improve TB care in T2D patients (Donovan et al. 2017).

Patients with TB-T2D comorbidity are more likely to fail TB treatment and present with more severe TB disease compared to TB patients without co-morbidities (Dooley et al. 2009), despite higher circulating levels of Th1 and Th17 cytokines in TB-T2D patients (Kumar et al. 2013). IL-10 is also elevated in TB-T2D patients (Kumar et al. 2013), however stimulation of whole blood from TB-T2D patients with PPD, showed higher Th1 associated cytokine profile but not Th2 cytokines (Restrepo et al. 2008). The elevated Th1 associated cytokines produced in TB-T2D potentially contributes to increased immune pathology (Kumar and Babu, 2017).

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Therefore we wanted to determine gene expression in the TB patients with and without T2D in South African Coloured population in an attempt to describe the immunological mechanisms associated with TB pathology and treatment outcomes. We identified gene transcripts including CCL2, CCL4, CCL3, IL-1A, CD163, CXCL2, PLAU, CXCL1, IL-1R1, TNFRSF8, CCRL2, NFKB1, PSMD7, PSMB5 and BAX, were downregulated in unstimulated whole blood of TB-T2D compared to both TB and TB-THG. MAPKAPK2 was upregulated in unstimulated whole blood of TB-T2D and TB-THG compared to TB. The downregulation of the following important chemokine gene transcripts; CCL2, CCL4, CCL3, CCRL2, CXCL1, CXCL2 may suggest alterations in leukocyte diapedesis and therefore alterations in the recruitment of immune cells to the site of inflammation. It has been shown that altered serum chemokine protein concentration can alter anti-TB immunity in T2D patients and can fuel TB-T2D (Aravindhan et al. 2018). The recruitment of phagocytic cells like neutrophils and macrophages are required to control Mtb growth and replication and initiate wound healing (Hong et al. 2004). Defects in trafficking of phagocytic cells to the site of inflammation will therefore delay innate and adaptive responses.

CCL2 acts as chemo-attractant for monocytes, as well as some other immune cells such as memory T lymphocytes and NK cells (Lu et al. 1998; Deshmane et al. 2009). CCL2 treatment of diabetic mice has shown immune cell infiltration into wounds which favours the healing of the wounds (Wood et al. 2014). CCL2 mRNA expression was significantly higher in the lungs of HN878-infected mice and associated with increased macrophage localization within the granuloma, however, in CCR2 knock out mice there was inefficient migration of macrophages to the granuloma when less CCL2 mRNA was expressed (Algood et al. 2003; Dunlap et al. 2018). We found that CCL2 was reduced in unstimulated whole blood of TB-T2D compared to TB and TB-THG. Infection of PBMCs with Mtb induced mRNA expression of CCL2 that was greater in pulmonary TB patients compared to localized extra pulmonary TB patients (Hasan et al. 2009).

Chemokines such as CCL3 released by neutrophils recruit monocytes to the site of infection (Mayadas et al. 2014). CCL3 mRNA was reported to be downregulated in whole blood of TB patients when compared to individuals with LTBI (Mistry et al. 2007). In addition, after infecting PBMCs with Mtb, there was no observed differences in CCL3 mRNA transcript among pulmonary TB, localised extra pulmonary TB and disseminated extra pulmonary groups (Hasan et al. 2009). Mtb infected mice were also reported to have low CCL3 mRNA compared to normal mice (Schneider et al. 2010). In support of these finding, we showed downregulation of CCL3 mRNA in unstimulated whole blood from TB-T2D compared to TB patients.

CCRL2 is a chemokine receptor also known as lipopolysaccharide (LPS) inducible C-C related gene (L-CCR) and shares the highest homology with CCR1 and CCR5 (Shimada et al. 1998;

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Otero et al. 2010). CCRL2 binds chemerin which is a chemotactic protein (Zabel et al. 2008). A previous study reported an increase in the expression of CCRL2 in the RAW 264.7 macrophage cell line and in murine macrophages after stimulation with LPS, this study was aimed at understanding the role of CCRL2 during microbial infection (Shimada et al. 1998). Stimulation of mouse neutrophils with LPS resulted in the increased expression of CCRL2, with most of the cells co-expressing CCRL2 and CXCR2 (Del Prete et al. 2017). To determine the role of CCRL2, mice deficient in CCRL2 were injected intraperitoneally with LPS and a reduction in neutrophil count was reported (Del Prete et al. 2017). In our study, we found CCRL2 mRNA to be downregulated in unstimulated whole blood of TB-T2D compared to TB. The difference in the study design, the use of cell lines and different antigen stimulants makes it difficult to speculate what effect the changes in CCRL2 mRNA will have in our study participants. There is however no information available on the expression of CCLR2 during TB-T2D. Since CCRL2 binds chemerin and presents it to the leukocytes expressing chemR23 (Gonzalvo-feo et al. 2014), the reduction in CCRL2 may be associated with reduced migration of leukocytes to inflammatory sites.

CXCL1 has been reported to be important for Th17 differentiation, neutrophil extracellular trap formation and reactive oxygen species production (Jin et al. 2014). Together, CXCL1 and CXCL2 have been identified as novel paracrine regulators for the production of anti-microbial molecules and inflammatory mediators during Mtb infection in humans (Boro et al. 2016). CXCL1 and CXCL2 are involved in the activation of inflammasomes and production of IL-1β in macrophages. When CXCL1 and CXCL2 were blocked using neutralizing antibodies, there was a reduction in IL-1β in supernatants after stimulation of macrophages with Mtb (Boro et al. 2017). In bone marrow derived (BMD) DCs from mice infected with BCG, large amounts of CXCL1 and CXCL2 were reported and resulted in an increased recruitment of neutrophils in the lungs of the mice (Doz et al. 2013). Neutrophils were subsequently responsible for the production of IL-10 and reduction in IL-17A production. Furthermore, depletion of the neutrophils resulted in a decrease in IL-10 and favoured IL-17A production (Doz et al. 2013).

CXCL1 and CXCL2 are expressed at low concentrations in the sera and the lung tissue of mice infected with Mtb (Schneider et al. 2010). Stimulation of polymorphonuclear granulocytes with Mtb and LAM resulted in increased mRNA and protein of CXCL1 by human polymorphonuclear granulocytes (Riedel and Kaufmann, 1997). BMD macrophages from mice infected with the Erdman Mtb strain had gene expression of CXCL2 (Scott and Flynn, 2002). In addition, infected BMD macrophages with the Erdman Mtb strain as well as the clinical isolates CSU22 and CSU46 also resulted in increased CXCL2 gene transcript expression (Rhoades et al. 1995). An increase in CXCL2 was also seen in a DM mice (db/db) compared to a control mice, three days post bacterial infection other than Mtb (Naguib et al. 2004). We

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found a downregulation of CXCL1 and CXCL2 gene transcript in unstimulated whole blood of TB patients. There is however no gene expression data available when comparing TB-T2D and TB patients.

CD163 is a scavenger receptor expressed exclusively on monocytes and macrophages, and function as an innate immune sensor for bacteria and induces local inflammation (Kristiansen et al. 2001). Increased CD163 expression on the surface of monocyte is associated with insulin resistance in patients with T2D, suggesting a novel pathophysiological role for CD163 (Kawarabayashi et al. 2017). CD163 mRNA was found to be higher in fat depots of obese patients compared to non-obese individuals (Kračmerová et al. 2014). Increased soluble CD163 was reported to be a strong predictor of risk for developing T2D in obese patients (Zanni et al. 2012). A positive correlation between plasma soluble CD163 and mRNA CD163 was described in the adipose tissue of obese patients (Kračmerová et al. 2014), however, CD163 frequency on MNs from adipose tissue was significantly decreased and soluble CD163 significantly increased in T2D patients with vascular complications compared to patients without vascular complications (Min et al. 2016). Suggesting that CD163 protects T2D patients from developing T2D associated complications (Min et al. 2016). CD163 protein therefore possibly plays a role in the pathophysiology of T2D.

In this study, we found that CD163 mRNA in TB-T2D was downregulated in unstimulated whole blood compared to TB patients. The upregulation of CD163 is a marker of switching from pro-inflammatory towards anti-inflammatory profile (Weaver et al. 2013), therefore, the downregulation of CD163 mRNA in TB-T2D corresponds to the pro-inflammatory profile in these patients. CD163 was also found to be significantly reduced on the surface of MNs from T2D patients after stimulation with LPS compared to unstimulated MNs (Khondkaryan et al. 2018), and was thought to be associated with persistent activation of MNs (Khondkaryan et al. 2018). In our study, we showed that stimulation of whole blood with Mtb-antigens, favours an Mtb specific upregulation of CD163 mRNA shifting responses to an anti-inflammatory profile in TB-T2D upon stimulation when compared to TB patients. The exact role of CD163 in chronic inflammation remains poorly described (Kawarabayashi et al. 2017) and requires further investigation. In our study, the downregulation of CD163 mRNA may be associated with immunopathology in TB-T2D patients.

Mtb promotes its replication by inhibiting apoptosis of infected macrophages (Blomgran et al. 2012). In our study, gene transcripts from unstimulated blood that plays a role in apoptosis (IL-1A, IL-1R1, TNFRSF8, NF-KB, and BAX) were lower in TB-T2D compared to TB. NF-kB is the transcription factor in classical macrophages responsible for the development of the Th1 response including the production of IL-12, IFN-γ, IL-23, and IL-27 which are required to

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control Mtb replication (Cooper and Khader, 2008; Liu et al. 2017), as well as IL-1β, TNF-α, and IL-6 (Wang et al. 2014).

S100A8, TAP1, S100A9, GBP1, TLR2, PSMB8 and PSMB9 were reported to be upregulated in whole blood of active TB patients compared to individuals with LTBI (Mistry et al. 2007). We also found that TAP1, TLR2, S100A9 and S100A8 are upregulated in unstimulated whole blood of TB compared to TB-T2D patients. S100A8 and S100A9 are upregulated during infection, stress, and many other inflammatory conditions (Wang et al. 2018). The increased expression of TLR (a receptor for pathogen associated molecules), in TB patients, shows that there is an upregulation of antigen recognition related pathways. The upregulation of TAP1 and protein subunits of proteasomes, all are related to antigen presentation signalling, which shows that during TB-T2D, these pathways may be less represented compared to TB.

PSMB5 along with C1R and C1QBP form part of the complement cascade and were collectively part of a gene signature that could distinguish TB-THG from TB-T2D in unstimulated whole blood. The complete cascade, as seen by C1R and C1QBP, as well as the antigen presentation pathway are the key pathways that are different in TB-THG and TB-T2D. The activation of C1R is associated with immune response pathways suggestive of any infectious disease (Wu et al. 2017). However, our study did not show differences in C1R mRNA between the patients. C1QBP was amongst the gene transcripts significantly downregulated in TB patients compared to LTBI in whole blood using microarrays (Mistry et al. 2007). We found, C1QBP to be downregulated in unstimulated whole blood of TB compared to TB-THG. In breast cancer the expression of PSMB5 mRNA was observed in M2 macrophages, and it was hypothesised to be involved in immunosuppressive responses (Wang et al. 2017). Knock down of PSMB5 allowed macrophages to differentiate into M1 phenotype. In our study, both PSMB5 and CD163 were downregulated in unstimulated whole blood of TB-T2D compared to TB.

6.1.2 Gene expression in LTBI with and without T2D

Blood transcriptional assays that enable early accurate TB diagnosis could have an important impact on control of the global TB epidemic (Walter et al. 2016). The immunological factors involved in the host immune response network in Mtb infection and LTBI reactivation have not yet been clearly elucidated. Identifying the host genes responsible for TB susceptibility and resistance may be the key towards better understanding the mechanisms leading to the development of the disease and identifying pathways that could be used as potential targets for HDT. The immune profile in LTBI-T2D is characterized by diminished production of Th1 cytokines. Diminished concentration of the Th1 cytokines allows for potential immunological mechanisms that could account for the increased risk for TB progression (Kumar and Babu. 2017). The immune profile in LTBI with T2D compared to LTBI without T2D is characterized

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by low Th1 (IFN-y, IL-2 and TNF-α) and Th2 but not Th17 associated cytokines which was partially modulated by TGF-β, since blocking TGF-β resulted in increased Th1 and Th2 cytokines (Kumar et al. 2016). There are however no gene expression data available from individuals with LTBI with T2D prior to developing active disease and TB-T2D. We therefore wanted to determine the differential gene expression in individuals with LTBI with and without T2D to identify host genes and pathways that could be associated with increased susceptibility to TB progression in LTBI-T2D. The genes that were differentially expressed in unstimulated whole blood of LTBI-T2D compared to LTBI were associated with the following functions or pathways: activation of necrosis and cell death of tumour cells, inhibition of immune response of leukocytes and maturation of blood cells of which some will impact immune response against Mtb. CCL2, CD163, TNFRSF8, CISH, GBP5, PML and TAP1 were differentially expressed in LTBI-T2D compared to LTBI and TB-T2D compared to TB patients and could potentially be involved in the underlying mechanisms responsible for the increased susceptibility to Mtb and the increased chance of developing active TB.

A three gene signature unique to LTBI-T2D was identified which includes CD59, CD274 and TNFSF11. Upon univariate analysis, only CD59 was found to be significantly different between the two groups and was higher in LTBI-T2D compared LTBI. The role of CD59 has been shown to protect host cells from the membrane attach complex mediated lysis (Koski et al. 1996). The membrane attack complex can result in tissue injury during dysregulated complement activation that occurs during chronic inflammatory conditions (Huang et al. 2006). This can result in overexpression of CD59, however overexpression of CD59 in tumour cells has been associated with tumour development (Fishelson et al. 2003), protecting tumour cells from monoclonal antibody treatment (Fishelson et al. 2003). CD59 plays a role in promoting tumour cell growth (Fishelson et al. 2003). A study by Cliff et al. investigated changes in gene expression before and during TB treatment in whole blood (Cliff et al. 2013). Among other gene transcripts, CD59 was upregulated at diagnosis of TB (Cliff et al. 2013). There is no information on CD59 in LTBI and T2D. In our LTBI-T2D group, CD59 may help Mtb evade immune responses by protecting the Mtb infected cells from complement mediated lysis and promote Mtb replication.

CD274, also referred to as programmed death 1 (PD-1), is a key immune checkpoint receptor expressed by activated T cells and mediates immune suppression (Yin et al. 2014). PD-1/PD-L1 interaction is known to inhibit T cell effector function during TB disease (Shen et al. 2016). Wang et al. reported that when PD-L1 and PD-L2 were upregulated, T cell associated gene transcripts were down regulated, which indicates that PD-Ls mediated suppression may be initiated during TB disease (Wang et al. 2018). PD-1 was shown to induce apoptosis of IFN-γ producing NKT cells while sparing NKT cells that produce IL-4. Such a polarized NKT cell

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function may impose a Th2 bias on the ensuing effector T cell response leading to inefficient clearance of Mtb. Inhibiting PD-1 may therefore affect the T cell response favouring the host by rescuing IFN-y producing NKT cells from apoptosis and enhancing Th1 effector T cell functions against Mtb (Singh et al. 2014). Phagocytosis and intracellular killing activity of macrophages were found to be increased significantly after blocking PD-1/PD-L pathway (Shen et al. 2016). Anti-PD-1/PD-L1 therapy holds promise as adjunctive therapy for chronic infectious diseases such as TB and should be tested in randomized clinical trials (Rao et al. 2017). In our study, PD-L1 transcript was upregulated in the LTBI-T2D patients in the unstimulated samples compared to LTBI. PD-L1 was also identified as one of the transcripts in the signature that could be associated with susceptibility to TB progression in LTBI with T2D by suppressing T cell immunity.

TNFSF11 encodes for receptor activator of the nuclear factor kappa B ligand (RANKL), which participates in physiological processes including organ development, bone remodelling and calcium homeostasis through osteoblast formation (Griffith et al.2012). There is no existing literature on the expression of TNFSF11 in individuals with LTBI. Our study did not show a significant difference in TNFSF11 expression between LTBI-T2D and LTBI, but in combination with other markers, can help us understand the mechanisms associated with TB susceptibility. RANKL has been reported to harbour immunosuppressive functions; blocking RANKL however, was not shown to affect immune responses in mice or humans (Guerrin et al. 2015; Conte et al. 2015). These findings suggest that RANKL plays a less prominent role in host defences against pathogens (Guerrini et al. 2015). In T2D, RANKL protein was upregulated compared to healthy patients (Conte et al. 2015). The role of RANKL has been studied in bone diseases and was found that a reduction in RANKL is associated with Mtb *H37Ra* induced bone loss. Whether this is enough to implicate TNFSF11 in the pathogenesis of T2D and susceptibility to TB progression is not clear.

6.1.3 Immune and endocrine changes and association with Mtb uptake and killing

Low-grade inflammation in T2D patients leads to the activation of the HPA-axis which subsequently results in increased cortisol secretion by the adrenals. Elevated levels of cortisol in serum result in immunosuppression leading to reduced Mtb containment (Pace et al. 2012). A few studies have focused on TB-T2D comorbidity (van Crevel and Dockrell, 2014; Fernandez et al. 2016). However, to date only a few studies in individuals with LTBI and T2D have been carried out (Kumar et al. 2013). We investigated the immune-endocrine profile to determine the effect of T2D on immune responses in recently Mtb exposed participants within the South African coloured population from the Western Cape. During an infection such as Mtb, the human host amounts an acute phase response (Gruys et al. 2005). These include changes in lipid profiles, cytokines, complement cascade and an increase in ACTH and

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glucocorticoid concentrations (Gruys et al. 2005). Comparison of acute phase protein responses and sympathetic as well as stress hormones such as catecholamines and cortisol in plasma of TB patients and controls was done (Opolot et al. 2015). It was reported that both cortisol and dopamine were higher in TB patients, although not significantly for cortisol (Opolot et al. 2015). Higher dopamine and lower adrenaline concentration in the TB group was reported compared to HCs. Previously, elevated concentrations of adrenaline, noradrenaline, dopamine and cortisol were associated with severity of TB disease (Hafeiz et al. 1992). Noradrenaline was reported to be higher in TB patients compared to controls (Opolot et al. 2015). We were unable to analyse the noradrenaline results due to technical errors. Mtb-induced responses feedback to the brain to orchestrate sympathetic nervous system responses which in turn mediate immune responses in the periphery. The same immune cells are then also a source of noradrenaline that induces early differentiation of Th1 cells essential for protection against mycobacterial infection (Alaniz et al. 1999). Mice genetically deficient of the enzyme dopamine-hydroxylase which is required for the synthesis of noradrenaline were found to be more susceptible to Mtb (Alaniz et al. 1999), suggesting that it participates in the generation of an optimal, protective cell-mediated immune response in vivo.

Noradrenaline was reported to be produced during early infection with Mtb by adrenergic nerve terminals and lymphocytes located in pulmonary inflammatory infiltrates and mediastinal lymph nodes, while during late infection noradrenaline production sharply decreases (Barrios-payán et al. 2016). This suggests that during advanced disease, the sympathetic nervous system activity is less important, or perhaps contributes to establishing local pro-inflammatory activity due to the absence of the anti-inflammatory effect of noradrenaline on differentiated immune cells (Pongratz and Straub, 2014). There was no differences in the catecholamines reported between our study groups. No other studies have reported on catecholamines in the context of T2D, and makes our study the first to report this.

Cytokines and hormones influence the cellular immune response, therefore the relationship and profile of plasma hormones such as cortisol needs to be investigated to understand how they affect immune response. In the plasma of TB patients compared to LTBI and HCs, concentration of cortisol was reported to be elevated (Santucci et al. 2011). In addition, cortisol levels were reported to be high in plasma of TB and TB-T2D patients compared to HCs (Fernandez et al. 2016). No difference have been reported between cortisol levels in TB and TB-T2D patients. The concentration of cortisol has also never been investigated in individuals with LTBI with and without T2D. We found no difference in cortisol in our study participants. Cortisol can inhibit Th1 responses, whereas DHEA induces them (Besedevoski et al. 1998; Turnbull et al. 1999). The HPA axis, represent a well-conserved mechanism to control an intense immune-inflammatory reaction as well as for the early mobilization of immune cells

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and their redistribution to mount an adequate defensive response (Attilio et al. 2018). During an immune response, cortisol can inhibit the proliferation of the effector and differential apoptosis of regulatory T cells (Pandolfi et al. 2013).

The upregulation of IL-6 is associated with inflammatory diseases such as TB (Martinez et al. 2013) and involved in metabolic disorders such as T2D (Liu et al. 2016). In TB-T2D, IL-6 plasma levels are high compared to TB (Kumar et al. 2013). In LTBI-T2D we also found IL-6 serum concentrations to be higher compared to LTBI. High IL-6 concentrations in culture supernatants of monocyte-derived macrophages (MDMs) of TB-T2D patients compared to TB patients (Lope-Lopez et al. 2018). IL-6 has the ability to impair insulin signalling in insulin-sensitive tissues (Nikolajczyk et al. 2012). High IL-6 concentrations has been shown to cause insulin resistance and T2D (Kumar et al. 2014). In our study, however, we did not calculate insulin resistance. Lower IL-6 production by macrophages from DM mice infected with *Mycobacterial fortuitum* was reported to contribute to increased bacterial growth in the infected macrophages, which suggest that IL-6 is required for better control of bacteria by macrophages (Abdul Alim et al. 2017). Plasma IL-6 concentrations were reported to be significantly higher in TB patients and TB-T2D patients compared to HCs (Fernandez et al. 2016). Despite the fact that IL-6 is pro-inflammatory and plays an important role in bacterial control, high expression in the serum from our LTBI-T2D study participants was associated with poor Mtb uptake by PBMCs. Contrary to these findings, Zheng et al. found no difference in IL-6 concentrations in TB patients with and without T2D and HCs, (Zheng et al. 2013). In plasma of individuals with LTBI compared to HCs, IL-6 was elevated (Santucci et al. 2011). Our data and data from other human studies, suggest that during T2D, IL-6 is elevated and that TB augments the levels of this cytokine. In humans, IL-6 is associated with pathogenesis, and reducing IL-6 production may restore insulin resistance and the function of beta-cells, reducing heart failure and fatigue in patients with T2D (Conti et al. 2018).

IL-18 plays a critical role by inducing Th1 responses during Mtb infection (Schneider et al. 2010; Ponnana et al. 2017). Without IL-18, Th1 responses are decreased and mycobacterial replication increases (Schneider et al. 2010). Mice deficient in IL-18 will succumb to Mtb infection (Sugawara et al. 1999; Schneider et al. 2010), and have suppressed IFN-γ production (Dinarello, 1999). IL-18 was low in TB-T2D patients compared to TB (Restrepo et al. 2008; Kumar et al. 2013). IL-18 induces the release of IL-8 from mononuclear cells (Puren et al. 1998), which is an attractant for neutrophils and also T cells (Puren et al. 1998). Neutrophilic inflammation is associated to TB pathogenesis (Berry et al. 2010) and, for this reason, we believe that IL-18 is involved in both protective and pathological responses. Liu et al. has shown that along with IL-1β and IL-6 elevated levels of IL-18 is associated with T2D (Liu et al. 2016). It is possible that IL-18 has different roles during LTBI with and without T2D. In contrast

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to what we found, Kumar et al. found serum IL-18 concentrations to be lower in LTBI-T2D (Kumar et al. 2014).This contradicting data suggests that more studies are required to evaluate the role of IL-18 in LTBI during T2D in different population groups.

IL-22 cytokine is produced by cells of the innate and adaptive immune system (Wolk et al. 2006), its role during host defence against Mtb infection still needs further investigation (Ronacher et al. 2018). Decreased plasma concentration of IL-22 was profoundly associated with susceptibility to impaired fasting glucose and T2D in Chinese subjects (Shen et al. 2018). In T2D foot ulcers, IL-20 subfamily including IL-22 are upregulated during normal wound healing (Kolumam et al. 2017). In addition, mice deficient in the receptor for IL-22 was associated with delay in the healing of the wounds. IL-22 was also associated with increased innate host immune defences and tissue remodelling in the skin (Kolumam et al. 2017). We did not find any significant differences in IL-22 serum concentrations between LTBI and LTBI-T2D, but we found differences between LTBI-T2D and LTB-pT2D, where IL-22 was significantly higher in LTBI-pT2D patients. Although the role of IL-22 has been associated with protection, excessive IL-22 responses may be detrimental. In fact, Ronacher et al. indicated that it is not clear whether IL-22 is increased to counteract immunopathology or if it contributes to pathology (Ronacher et al. 2018). Kumar et al. has reported higher IL-22 concentrations in LTBI-T2D compared to LTBI (Kumar et al. 2015), whereas we did not observe any significant differences between the two groups. The difference between the two studies being that Kumar et al. did not categorize the T2D patients into the different subgroups e.g. pre-T2D, T2D and pT2D. Comparison of circulating cytokines between individuals with LTBI with high BMI and low BMI was done and found higher IL-22 concentrations in high BMI individuals with LTBI (Anuradha et al. 2016), suggesting a link between BMI and IL-22 production. In our study, the individuals with LTBI with and without T2D did not show any significance difference in BMI, and perhaps could be why we did not see any difference in IL-22 concentrations.

We observed significantly higher IL-22 concentrations in TB-T2D patients compared to TB-THG at BL and no difference was observed between TB-T2D and TB patients. This is different to what Kumar et al. found, where IL-22 was lower in plasma samples of TB-T2D compared to TB (Kumar et al. 2013; Kumar et al. 2015). A negative correlation has been observed between IL-22 expression in the periphery and the lung, with higher IL-22 measured in BALF (Singh et al. 2018). Since serum IL-22 was higher in our TB-T2D patients, it is possible that these patients have low IL-22 in the lung where it is needed.

Vitamin D deficiency is associated with an increased risk of TB disease and poor treatment outcome (Kim et al. 2017). When MDMs from T2D patients with low vitamin D receptor expression were supplemented with vitamin D, they were able to eliminate Mtb efficiently (Lopez-lopez et al. 2014). This study suggests the use of vitamin D as prophylaxis for TB in

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high T2D endemic countries (Lopez-lopez et al. 2014). vitamin D in serum of patients with history of TB disease compared to uninfected controls was significantly lower vitamin D concentration in individuals with a history of TB compared to uninfected individuals (Kim et al. 2018). In T2D patients, the same has also been reported where vitamin D is significantly reduced in their serum compared to HCs (Chandra et al. 2004). The high vitamin D in HCs, may partly be involved in the significantly enhanced phagocytic potential of macrophages isolated from these individuals (Chandra et al. 2004).

Dyslipidaemia in T2D is characterized by high levels of triglycerides, and/or cholesterol as well as low HDL (Vrieling et al. 2018). In addition, dyslipidaemia is associated with necrotic granulomas and fibroplasia leading to exacerbated lung damage in TB patients and especially in TB-T2D patients (Dong et al. 2018). Dong et al. reported low HDL in smear negative patients with TB-T2D, which was associated with severe lung lesions in these patients (Dong et al. 2018). HDL serum levels are reduced during infections and inflammation (Deniz et al. 2006; Dong et al .2018). This agrees with what we found where HDL concentrations were lower in LTBI-T2D compared to individuals with LTBI. Since low HDL is associated with severe lung lesions in TB-T2D patients as reported by Dong et al. and hyperinflammatory response of TB-T2D (Prada-medina et al. 2017), one can assume that low concentrations in individuals with LTBI and T2D may play a role in susceptibility to TB disease. Cholesterol serves as an important carbon source utilized by Mtb to growth and persistence in mice (Pandey and Sassetti, 2008). High CHL has been identified as a risk factor for TB disease (Martens et al. 2008; Soh et al. 2016) and statins use, which lowers CHL concentrations, reduces the risk of developing TB disease (Lai et al. 2016; Su et al. 2017). A potential therapy targeting CHL catabolism can effectively lower intracellular growth of Mtb (Tsai et al. 2017) and more studies should focus on investigating the use of cholesterol lowering drugs in conjunction with TB treatment to prevent TB disease in groups that are at increased risk such as LTBI-T2D. Lipids, such as LDL, may stimulate macrophage phagocytosis and subsequent release of inflammatory markers, induce mast cell degranulation and leukocyte recruitment (Medina et al. 2015). Our study did not show any relationship between LDL and Mtb killing, but in the larger parent study, the ALERT study, we found that LDL was associated with reduced Mtb growth in MNs (Manuscript in preparation). This shows that LDL can induce response against Mtb possible by facilitating the killing of Mtb in immune cells.

Macrophages play a central role in the initiation and resolution of inflammatory responses to infectious pathogens (Auffray et al. 2009) and are frequently activated in T2D patients (Bianciardi, 2015), which means that they could play an important role in T2D development. Macrophages are equipped with scavenger receptors that recognize lipids and various microorganisms (Auffray et al. 2009). The high incidence of infectious diseases in patients

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with T2D (Peleg et al. 2007; Weintrob et al.2009) indicates a dysfunctional innate immune response in these patients. In fact, reduced phagocytic and chemotactic activity of MNs and neutrophils have been shown (Delamaire et al. 1997; Llorente et al. 2000; Restrepo et al. 2014). Our results supported previous studies where no difference in Mtb killing by MDMs, and PBMCs were observed (Reyes-ruvalcaba et al. 2008; Raposo-Garcia et al. 2017). However, it is possible that we do not see differences in the phagocytosis of MNs from T2D patients compared to noT2D because of small sample size, as data from the larger ALERT study, show differences in Mtb uptake by MNs from T2D compared to noT2D (Manuscript in preparation).

6.1.4 MAIT cells in TB patients with and without T2D

MAIT cells have innate like functions and have gained much attention as important role players in immunity to infectious diseases, such as Mtb, as well as in the pathogenesis of non-communicable diseases, such as T2D (Shey et al. 2018). Most MAIT cells are identified as $CD8^+$ and $CD4^-$, but a small subset of MAIT cells do express CD4 (Kurioka et al. 2015). The different MAIT cell subsets suggests that they may also have unique functions. Recently, it has been shown that riboflavin tetramers can be used to identify all MAIT cells (Reantragoon et al. 2013). MAIT cells can be activated through TCR dependent and independent pathway (Gold et al. 2010; Kurioka et al. 2015), regardless of the disease state. MAIT cells have site specific functions. The frequency of IL-17 producing MAIT cells were higher in the adipose tissue of T2D patients compared to the periphery (Magalhaes et al. 2015). Higher frequencies of MAIT cells producing IL-17 and IL-22 were found in the vaginal tract compared to higher frequencies of MAIT cells producing IFN-γ and TNF-α in the periphery (Gibbs et al. 2017).

At baseline, MAIT cells are significantly reduced in the periphery of TB patients compared to uninfected HCs (Gold et al. 2010; Wong et al. 2013) which recover during the course of TB treatment (Sharma et al. 2015; Wong et al. 2013). When MAIT cells are reduced in the periphery during TB disease they migrate to the lung, as shown by measuring MAIT cell frequency in the BALF of TB patients with and without HIV co-infection (Wong et al. 2015). In TB-HIV co-infected patients, treatment did not restore MAIT cell frequencies in the periphery compared to TB patients (Wong et al. 2013). It has been shown that TB-HIV patients have high viral load in the lung (Shankar et al. 2014), because of the presence of the virus the MAIT cells remain in the lung. Similarly, in our TB-T2D patients, failure of MAIT cells to increase at M6 of TB treatment could mean that MAIT cells are still present in the adipose tissue or the lung. Malherbe et al. showed that even at M6 of TB treatment, after TB patients are cured based on negative MGIT culture results, there is still lesions in the lung consistent with TB disease and Mtb specific mRNA (Malherbe et al. 2016), which means that MAIT cells could still be in the lung even though the patients are clinically cured. There are no published results

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on MAIT cell frequency in TB-T2D. Our study is the first to and provide information on the frequency of $CD8^{+}TCR\ 7.2^{++}CD161^{++}CD26^{++}$ MAIT cells and the influence of TB treatment on the frequency of these cells in TB and T2D patients.

6.2 Conclusion

Our first aim was to identify differentially expressed immune genes in unstimulated and Mtb-antigen stimulated whole blood of newly exposed LTBI positive participants as well as TB patients with and without T2D. To achieve this RNA was isolated from QFT stimulated blood and differential expression of 594 genes involved in immune regulation was determined with the NanoString technology.

Our study found fifteen important gene transcripts that need to be further investigated in the TB-T2D comorbidity to understand the immunological mechanisms involved in the pathology of T2D in TB patients. Proteins associated with these genes have been described, however, the true relationship between the protein and their gene transcripts have not been shown and this could give us a better understanding of what is happening during Mtb infection. We found genes associated with phagocytes trafficking to the site of infection, and those associated with apoptosis to be downregulated in the TB-T2D compared to TB and TB-THG. Furthermore, we were able to identify a gene signature that differentiates between THG and T2D in TB patients. This signature could be useful to identify THG and prevent the unnecessary treatment of these patients who would have been diagnosed with T2D. Most importantly, T2D screening of TB patients is recommended by the WHO, however HbA1c testing will not be able to distinguish patients with THG from T2D. Therefore, our gene signature could complement standard T2D screening of TB patients. We also identified a gene signature unique to individuals with LTBI with T2D. We show that these gene signatures are associated with complement pathways which may be involved in TB progression of individuals with LTBI-T2D.

Secondly, we aimed to investigate immune and endocrine changes in the serum and plasma of individuals with LTBI with and without T2D and determine their association with Mtb uptake and killing. Chronic activation of the innate system is associated with T2D, and abnormal production of cytokines and chemokines. In our study, Th1 cytokines and IL-22 which is produced by Th17 and/ Th22 are more pronounced in LTBI-pT2D, which showed a heighted pro-inflammatory cytokine profiles including IL-6, IL-18 and IL-22 compared to individuals with LTBI. Lipid production in the plasma was also increased. Our results adds to the small body of knowledge that is available on the production of immune products by cells of individuals with LTBI with and without T2D. We have shown that there is a need for more studies to define the immune profile of these individuals, to help us learn and understand the immune mechanisms during Mtb infection and influence of plasma soluble proteins and lipids in Mtb phagocytosis and killing assays.

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Lastly, we wanted to determine the frequency of MAIT cells in TB, TB-THG and TB-T2D at BL, M2 and M6 of TB treatment, since the reduction of MAIT cells in metabolic and infectious diseases have been widely shown. Despite their frequencies, the functions of MAIT cells in human TB-T2D have not be demonstrated. The varying frequencies of MAIT cells both during diseases and change during treatment, shows that they have an essential role during TB diseases and their roles needs to be delineated especially in patient with TB and T2D. Our results showed that the frequency of MAIT cells in TB-T2D was significantly lower compared to TB-THG at BL. This could mean that the MAIT cells in TB-T2D have redistributed to the tissue, either to the lung or the adipose tissue. The increase in the periphery at M2 and M6 compared to BL could mean that the number of MAIT cells were restored to the blood due to good treatment outcome and reduced Mtb in the lung. MAIT cells could be used as a potential subtype for monitoring treatment responses in TB-T2D patients.

Overall, with ALERT data we were able to show the link between the endocrine and the immune response. Although we could not show differences in cortisol levels as previously thought, the higher insulin production in plasma of LTBI-T2D as well as other cytokines such as IL-6, IL-18 and IL-22 from our study may reflect an association and connection between the immune and the neuroendocrine system. The reduced association of Mtb with PBMCs from individuals with LTBI-T2D as well as reduced Mtb control by MNs from LTBI-T2D may be linked to the insulin and cytokine levels in these individuals with LTBI-T2D and may in part play a role in the functioning of these immune cells. Insulin resistance has also been associated with increased Th1 and Th17 cytokine production. Even though Insulin has been shown to drive T cell differentiation towards an anti-inflammatory phenotype, we saw an increased insulin along with increased IL-6, IL-18 and IL-22 in our participants. These cytokines may also contribute to poor glycemic control and hyperlipidaemia as shown in our results where their increase was also accompanied by increased triglycerides in individuals with LTBI-T2D. Following this we will be measuring a range of hormones and cytokines directly from culture supernatants of the PBMCs and MNs infected with Mtb H37Rv to fully describe the interplay between mononuclear cells and hormones during T2D.

6.3 Strengths and limitations of the study

The number of study participants with LTBI with and without T2D was small. Recruitment of study participants continued beyond the time frame of my PhD, samples that were available at the time were used. This work continued to include all the participants enrolled in the study to increase the sample size and has now reached the recruitment target and analysis of data is ongoing. Serum cytokines and plasma hormone concentrations were correlated to the Mtb killing assay as they were cultured in 20% autologous plasma. As part of the larger study we

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will continue to measure the cytokine concentrations in the culture supernatants which will also be correlated to Mtb killing assays. For the TANDEM study, only a small number of TB-T2D were recruited, and matching the participants also resulted in even smaller numbers. We were not able to do Mtb killing assays using the PBMCs of TB-T2D patients since the stored cells were not enough to do the killing assays. There were a limited number of samples available for the TANDEM study which prevented us from running all the ELISA and luminex assays on these participants. We were able to perform gene expression assay on both TANDEM and ALERT samples. We were not able to perform a direct comparison for our 594 gene expression between the participant groups of the two different studies as the gene expression analysis was batched and run at different times. The first gene expression analysis was performed on the TANDEM patients, and a year later we ran the same gene expression panel on ALERT patients. Seeing that there is limited information on MAIT cells in TB with and without T2D, therefore we wanted to measure MAIT cell frequency in TB and TB-T2D patients and include LTBI-T2D as a control, however, we ended up doing this without a LTBI-T2D control, since we did not have enough PBMCs for the larger part of these individuals. Another limitation to our study for both ALERT and TANDEM populations was the exclusion of HIV positive individuals seeing that TB-HIV is quite a concern in South Africa. In general, the use of HbA1c and FBG as a diagnostic for T2D without the support of OGTT was another limitation since the OGTT is considered the gold standand and has been shown to perform as best diagnostic for T2D in our study population (Zemlin et al. 2011).

6.4 Future work

The identified genes signature that distinguish between TB-THG and TB-T2D need to be validated by screening for these signature in a different cohort so that it can be incorporated in a simple test to be used together with HbA1c to describe true T2D patients. The identified gene signature in latent TB with T2D will be followed up and further explored using functional assays, to understand the role of the transcripts and resulting proteins during Mtb infection. Cytokines and hormones are going to be measured from monocytes and PBMC culture supernatants and to identify associations between Mtb uptake and killing. This will give us a better understanding of what the immune and endocrine profile is during Mtb infection in individuals with LTBI with and without T2D and how that influence phagocytosis and Mtb killing. We collected and stored whole blood and broncho alveolar lavage from participants with LTBI with and without T2D, this will be used to determining the frequency of MAIT cells in the periphery and also at the site of TB disease, we would like to do this for TB with and without T2D patients as well to determine the frequency of MAIT cells recruited to the site of disease.

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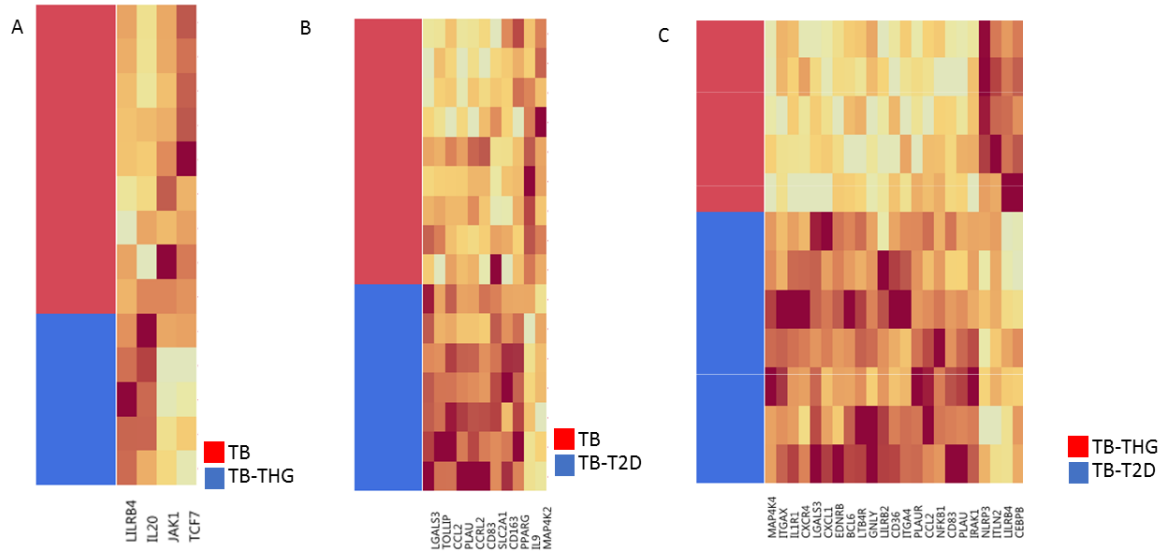
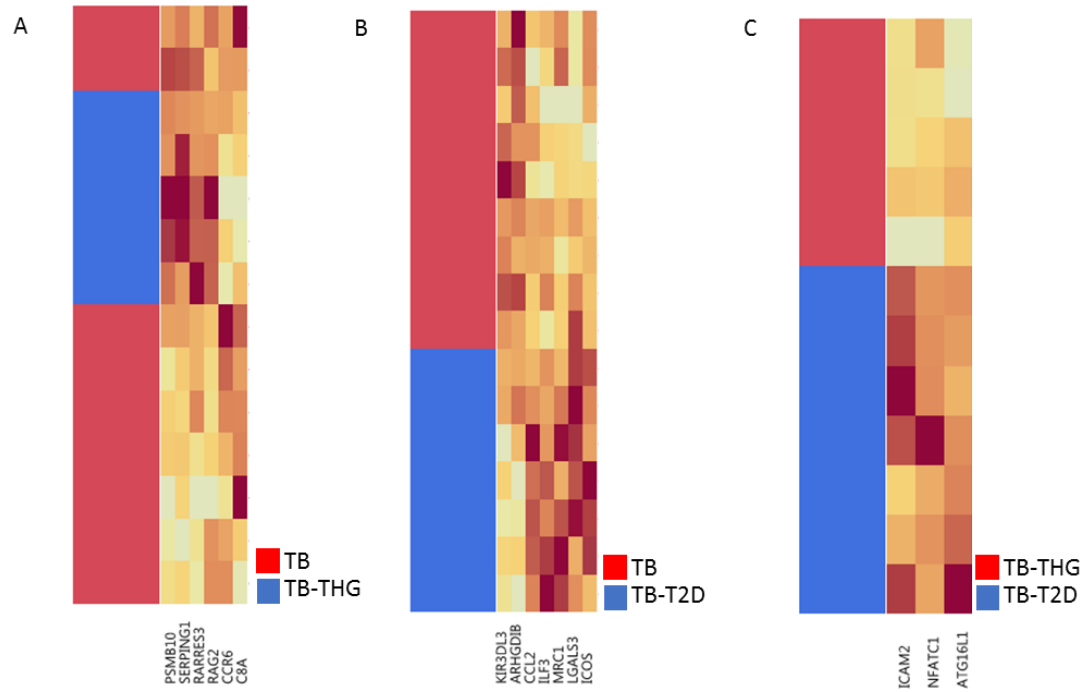
Addenda



Supplementary Figure 1: Differentially expressed gene transcripts between TB, TB-THG and TB-T2D. RNA was isolated from whole blood of TB-T2D, TB-THG and TB stimulated with Mtb specific antigen using QFT assay (Ag/nil, stimulated only represented in the figure). Gene expression of 594 genes as part of the nCounter GX Human Immunology kit V2 was done on the extracted RNA. Down and upregulated transcripts from unstimulated blood of TB compared to TB-THG (A), TB vs. TB-T2D (B) and TB-THG vs TB-T2D. JMP SAS was used to generate heatmaps and used Student T test to determine statistical difference between the groups. Genes with a p value was < 0.01 and FDR < 0.05 was statistically significant. Figure A Red TB, Blue TB-THG, Figure B red TB, blue TB-T2D and Figure C red TB-THG and blue TB-T2D.



Supplementary Figure 2: Differentially expressed gene transcripts between TB, TB-THG and TB-T2D at month 6. RNA was isolated from whole blood of TB-T2D, TB-THG and TB stimulated with Mtb specific antigen using QFT assay (Ag/nil, stimulated only represented in the figure). Gene expression of 594 genes as part of the nCounter GX Human Immunology kit V2 was done on the extracted RNA. Down and upregulated transcripts from unstimulated blood of TB compared to TB-THG (A), TB vs. TB-T2D (B) and TB-THG vs TB-T2D. JMP SAS was used to generate heatmaps and used Student T test to determine statistical difference between the groups. Genes with a p value was < 0.01 and FDR < 0.05 was statistically significant. Figure A Red TB, Blue TB-THG, Figure B red TB, blue TB-T2D and Figure C red TB-THG and blue TB-T2D.

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Supplemtary table 1: List of Nanostring genes included in the nCounter GX Human Immunology kit V2

Official Symbol	Accession	Official Symbol	Accession	Official Symbol	Accession	Official Symbol	Accession	Official Symbol	Accession
ABCB1	NM_000927.3	BST1	NM_004334.2	CCL11	NM_002986.2	CD163	NM_004244.4	CD58	NM_001779.2
ABL1	NM_005157.3	BST2	NM_004335.2	CCL13	NM_005408.2	CD164	NM_006016.4	CD59	NM_000611.4
ADA	NM_000022.2	BTK	NM_000061.1	CCL15	NM_032965.3	CD19	NM_001770.4	CD6	NM_006725.3
AHR	NM_001621.3	BTLA	NM_181780.2	CCL16	NM_004590.2	CD1A	NM_001763.2	CD7	NM_006137.6
AICDA	NM_020661.1	C14orf166	NM_016039.2	CCL18	NM_002988.2	CD1D	NM_001766.3	CD70	NM_001252.2
AIRE	NM_000383.2	C1QA	NM_015991.2	CCL19	NM_006274.2	CD2	NM_001767.3	CD74	NM_001025159.1
APP	NM_000484.3	C1QB	NM_000491.3	CCL2	NM_002982.3	CD209	NM_021155.2	CD79A	NM_001783.3
ARG1	NM_000045.2	C1QBP	NM_001212.3	CCL20	NM_004591.1	CD22	NM_001771.2	CD79B	NM_021602.2
ARG2	NM_001172.3	C1R	NM_001733.4	CCL22	NM_002990.3	CD24	NM_013230.2	CD80	NM_005191.3
ARHGDIB	NM_001175.4	C1S	NM_001734.2	CCL23	NM_145898.1	CD244	NM_016382.2	CD81	NM_004356.3
ATG10	NM_001131028.1	C2	NM_000063.3	CCL24	NM_002991.2	CD247	NM_198053.1	CD82	NM_002231.3
ATG12	NM_004707.2	C3	NM_000064.2	CCL26	NM_006072.4	CD27	NM_001242.4	CD83	NM_004233.3
ATG16L1	NM_198890.2	C4A/B	NM_007293.2	CCL3	NM_002983.2	CD274	NM_014143.3	CD86	NM_175862.3
ATG5	NM_004849.2	C4BPA	NM_000715.3	CCL4	NM_002984.2	CD276	NM_001024736.1	CD8A	NM_001768.5
ATG7	NM_001136031.2	C5	NM_001735.2	CCL5	NM_002985.2	CD28	NM_001243078.1	CD8B	NM_004931.3
ATM	NM_000051.3	C6	NM_000065.2	CCL7	NM_006273.2	CD34	NM_001025109.1	CD9	NM_001769.2
B2M	NM_004048.2	C7	NM_000587.2	CCL8	NM_005623.2	CD36	NM_001001548.2	CD96	NM_005816.4
B3GAT1	NM_018644.3	C8A	NM_000562.2	CCND3	NM_001760.2	CD3D	NM_000732.4	CD97	NM_078481.2
BATF	NM_006399.3	C8B	NM_000066.2	CCR1	NM_001295.2	CD3E	NM_000733.2	CD99	NM_002414.3
BATF3	NM_018664.2	C8G	NM_000606.2	CCR10	NM_016602.2	CD3EAP	NM_012099.1	CDH5	NM_001795.3
BAX	NM_138761.3	C9	NM_001737.3	CCR2	NM_001123041.2	CD4	NM_000616.4	CDKN1A	NM_000389.2
BCAP31	NM_005745.7	CAMP	NM_004345.3	CCR5	NM_000579.1	CD40	NM_001250.4	CEACAM1	NM_001712.3
BCL10	NM_003921.2	CARD9	NM_052813.4	CCR6	NM_031409.2	CD40LG	NM_000074.2	CEACAM6	NM_002483.4
BCL2	NM_000657.2	CASP1	NM_001223.3	CCR7	NM_001838.2	CD44	NM_001001392.1	CEACAM8	NM_001816.3
BCL2L11	NM_138621.4	CASP10	NM_032977.3	CCR8	NM_005201.2	CD46	NM_172350.1	CEBPB	NM_005194.2
BCL3	NM_005178.2	CASP2	NM_032982.2	CCRL1	NM_016557.2	CD48	NM_001778.2	CFB	NM_001710.5
BCL6	NM_001706.2	CASP3	NM_032991.2	CCRL2	NM_003965.4	CD5	NM_014207.2	CFD	NM_001928.2
BID	NM_001196.2	CASP8	NM_001228.4	CD14	NM_000591.2	CD53	NM_001040033.1	CFH	NM_001014975.2
BLNK	NM_013314.2	CCBP2	NM_001296.3	CD160	NM_007053.2	CD55	NM_000574.3	CFI	NM_000204.3
CFP	NM_002621.2	CXCL1	NM_001511.1	FCER1A	NM_002001.2	HLA-DMA	NM_006120.3	IFNG	NM_000619.2
CHUK	NM_001278.3	CXCL10	NM_001565.1	FCER1G	NM_004106.1	HLA-DMB	NM_002118.3	IFNGR1	NM_000416.1

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CIITA	NM_000246.3	CXCL11	NM_005409.4	FCGR1A/B	NM_000566.3	HLA-DOB	NM_002120.3	IGF2R	NM_000876.1
CISH	NM_145071.2	CXCL12	NM_000609.5	FCGR2A	NM_021642.3	HLA-DPA1	NM_033554.2	IKBKAP	NM_003640.3
CLEC4A	NM_194448.2	CXCL13	NM_006419.2	FCGR2A/C	NM_201563.4	HLA-DPB1	NM_002121.4	IKBKB	NM_001556.1
CLEC4E	NM_014358.2	CXCL2	NM_002089.3	FCGR2B	NM_001002273.1	HLA-DQA1	NM_002122.3	IKBKE	NM_014002.2
CLEC5A	NM_013252.2	CXCL9	NM_002416.1	FCGR3A/B	NM_000570.4	HLA-DQB1	NM_002123.3	IKBKG	NM_003639.2
CLEC6A	NM_001007033.1	CXCR1	NM_000634.2	FCGRT	NM_004107.4	HLA-DRA	NM_019111.3	IKZF1	NM_006060.3
CLEC7A	NM_197954.2	CXCR2	NM_001557.2	FKBP5	NM_001145775.1	HLA-DRB1	NM_002124.2	IKZF2	NM_016260.2
CLU	NM_001831.2	CXCR3	NM_001504.1	FN1	NM_212482.1	HLA-DRB3	NM_022555.3	IKZF3	NM_183232.2
CMKLR1	NM_004072.1	CXCR4	NM_003467.2	FOXP3	NM_014009.3	HRAS	NM_005343.2	IL10	NM_000572.2
CR1	NM_000651.4	CXCR6	NM_006564.1	FYN	NM_002037.3	ICAM1	NM_000201.2	IL10RA	NM_001558.2
CR2	NM_001006658.1	CYBB	NM_000397.3	GATA3	NM_001002295.1	ICAM2	NM_000873.3	IL11RA	NM_147162.1
CRADD	NM_003805.3	DEFB1	NM_005218.3	GBP1	NM_002053.1	ICAM3	NM_002162.3	IL12A	NM_000882.2
CSF1	NM_000757.4	DEFB103A	NM_001081551.2	GBP5	NM_052942.3	ICAM4	NM_001039132.1	IL12B	NM_002187.2
CSF1R	NM_005211.2	DEFB103B	NM_018661.3	GFI1	NM_005263.2	ICAM5	NM_003259.3	IL12RB1	NM_005535.1
CSF2	NM_000758.2	DEFB4A	NM_004942.2	GNLY	NM_006433.2	ICOS	NM_012092.2	IL13	NM_002188.2
CSF2RB	NM_000395.2	DPP4	NM_001935.3	GP1BB	NM_000407.4	ICOSLG	NM_015259.4	IL13RA1	NM_001560.2
CSF3R	NM_156038.2	DUSP4	NM_057158.2	GPI	NM_000175.2	IDO1	NM_002164.3	IL15	NM_172174.1
CTLA4_all (common probe)	NM_005214.3	EBI3	NM_005755.2	GPR183	NM_004951.3	IFI16	NM_005531.1	IL16	NM_004513.4
CTLA4-TM (membrane-bound form)	NM_005214.3	EDNRB	NM_003991.2	GZMA	NM_006144.2	IFI35	NM_005533.3	IL17A	NM_002190.2
sCTLA4 (soluble form)	NM_001037631.1	EGR1	NM_001964.2	GZMB	NM_004131.3	IFIH1	NM_022168.2	IL17B	NM_014443.2
CTNNB1	NM_001098210.1	EGR2	NM_000399.3	GZMK	NM_002104.2	IFIT2	NM_001547.4	IL17F	NM_052872.3
CTSC	NM_001814.4	ENTPD1	NM_001098175.1	HAMP	NM_021175.2	IFITM1	NM_003641.3	IL18	NM_001562.2
CTSG	NM_001911.2	EOMES	NM_005442.2	HAVCR2	NM_032782.3	IFNA1/13	NM_024013.1	IL18R1	NM_003855.2
CTSS	NM_004079.3	ETS1	NM_005238.3	HFE	NM_139011.2	IFNA2	NM_000605.3	IL18RAP	NM_003853.2
CUL9	NM_015089.2	FADD	NM_003824.2	HLA-A	NM_002116.5	IFNAR1	NM_000629.2	IL19	NM_013371.3
CX3CL1	NM_002996.3	FAS	NM_000043.3	HLA-B	NM_005514.6	IFNAR2	NM_000874.3	IL1A	NM_000575.3

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CX3CR1	NM_001337.3	FCAR	NM_133280.1	HLA-C	NM_002117.4	IFNB1	NM_002176.2	IL1B	NM_000576.2
IL1R1	NM_000877.2	IL6ST	NM_002184.2	JAK1	NM_002227.1	LEF1	NM_016269.3	MAPKAPK2	NM_004759.3
IL1R2	NM_173343.1	IL7	NM_000880.2	JAK2	NM_004972.2	LGALS3	NM_001177388.1	MARCO	NM_006770.3
IL1RAP	NM_002182.2	IL7R	NM_002185.2	JAK3	NM_000215.2	LIF	NM_002309.3	MASP1	NM_139125.3
IL1RL1	NM_016232.4	IL8	NM_000584.2	KCNJ2	NM_000891.2	LILRA1	NM_006863.1	MASP2	NM_139208.1
IL1RL2	NM_003854.2	IL9	NM_000590.1	KIR_Activating_Subgroup_1	NM_001083539.1	LILRA2	NM_006866.2	MBL2	NM_000242.2
IL1RN	NM_000577.3	ILF3	NM_001137673.1	KIR_Activating_Subgroup_2	NM_014512.1	LILRA3	NM_006865.3	MBP	NM_002385.2
IL2	NM_000586.2	IRAK1	NM_001569.3	KIR_Inhibiting_Subgroup_1	NM_014218.2	LILRA4	NM_012276.3	MCL1	NM_021960.3
IL20	NM_018724.3	IRAK2	NM_001570.3	KIR_Inhibiting_Subgroup_2	NM_014511.3	LILRA5	NM_181879.2	MIF	NM_002415.1
IL21	NM_021803.2	IRAK3	NM_007199.1	KIR3DL1	NM_013289.2	LILRA6	NM_024318.2	MME	NM_000902.2
IL21R	NM_021798.2	IRAK4	NM_016123.1	KIR3DL2	NM_006737.2	LILRB1	NM_001081637.1	MR1	NM_001531.2
IL22	NM_020525.4	IRF1	NM_002198.1	KIR3DL3	NM_153443.3	LILRB2	NM_005874.1	MRC1	NM_002438.2
IL22RA2	NM_181310.1	IRF3	NM_001571.5	KIT	NM_000222.2	LILRB3	NM_006864.2	MS4A1	NM_152866.2
IL23A	NM_016584.2	IRF4	NM_002460.1	KLRAP1	NR_028045.1	LILRB4	NM_001081438.1	MSR1	NM_002445.3
IL23R	NM_144701.2	IRF5	NM_002200.3	KLRB1	NM_002258.2	LILRB5	NM_001081442.1	MUC1	NM_001018017.1
IL26	NM_018402.1	IRF7	NM_001572.3	KLRC1	NM_002259.3	LITAF	NM_004862.3	MX1	NM_002462.2
IL27	NM_145659.3	IRF8	NM_002163.2	KLRC2	NM_002260.3	LTA	NM_000595.2	MYD88	NM_002468.3
IL28A	NM_172138.1	IRGM	NM_001145805.1	KLRC3	NM_007333.2	LTB4R	NM_181657.3	NCAM1	NM_000615.5
IL28A/B	NM_172139.2	ITGA2B	NM_000419.3	KLRC4	NM_013431.2	LTB4R2	NM_019839.4	NCF4	NM_000631.4
IL29	NM_172140.1	ITGA4	NM_000885.4	KLRD1	NM_002262.3	LTBR	NM_002342.1	NCR1	NM_004829.5
IL2RA	NM_000417.1	ITGA5	NM_002205.2	KLRF1	NM_016523.1	LTF	NM_002343.2	NFATC1	NM_172389.1
IL2RB	NM_000878.2	ITGA6	NM_000210.1	KLRF2	NM_001190765.1	LY96	NM_015364.2	NFATC2	NM_012340.3
IL2RG	NM_000206.1	ITGAE	NM_002208.4	KLRG1	NM_005810.3	MAF	NM_005360.4	NFATC3	NM_004555.2
IL3	NM_000588.3	ITGAL	NM_002209.2	KLRG2	NM_198508.2	MALT1	NM_006785.2	NFIL3	NM_005384.2
IL32	NM_001012633.1	ITGAM	NM_000632.3	KLRK1	NM_007360.1	MAP4K1	NM_007181.3	NFKB1	NM_003998.2
IL4	NM_000589.2	ITGAX	NM_000887.3	LAG3	NM_002286.5	MAP4K2	NM_004579.2	NFKB2	NM_002502.2
IL4R	NM_000418.2	ITGB1	NM_033666.2	LAIR1	NM_002287.3	MAP4K4	NM_004834.3	NFKBIA	NM_020529.1
IL5	NM_000879.2	ITGB2	NM_000211.2	LAMP3	NM_014398.3	MAPK1	NM_138957.2	NFKBIZ	NM_001005474.1
IL6	NM_000600.1	ITLN1	NM_017625.2	LCK	NM_005356.2	MAPK11	NM_002751.5	NLRP3	NM_001079821.2
IL6R	NM_000565.2	ITLN2	NM_080878.2	LCP2	NM_005565.3	MAPK14	NM_001315.1	NOD1	NM_006092.1

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NOD2	**NM_022162.1**	**PSMC2**	**NM_002803.3**	**SH2D1A**	**NM_001114937.2**	**TCF7**	**NM_003202.2**	**TNFRSF4**	**NM_003327.2**
NOS2	NM_000625.4	PSMD7	NM_002811.3	SIGIRR	NM_021805.2	TFRC	NM_003234.1	TNFRSF8	NM_152942.2
NOTCH1	NM_017617.3	PTAFR	NM_000952.3	SKI	NM_003036.2	TGFB1	NM_000660.3	TNFRSF9	NM_001561.4
NOTCH2	NM_024408.3	PTGER4	NM_000958.2	SLAMF1	NM_003037.2	TGFBI	NM_000358.2	TNFSF10	NM_003810.2
NT5E	NM_002526.2	PTGS2	NM_000963.1	SLAMF6	NM_001184714.1	TGFBR1	NM_004612.2	TNFSF11	NM_003701.2
PAX5	NM_016734.1	PTK2	NM_005607.3	SLAMF7	NM_021181.3	TGFBR2	NM_001024847.1	TNFSF12	NM_003809.2
PDCD1	NM_005018.1	PTPN2	NM_002828.2	SLC2A1	NM_006516.2	THY1	NM_006288.2	TNFSF13B	NM_006573.4
PDCD1LG2	NM_025239.3	PTPN22	NM_015967.4	SMAD3	NM_005902.3	TICAM1	NM_014261.1	TNFSF15	NM_001204344.1
PDCD2	NM_144781.2	PTPN6	NM_002831.5	SMAD5	NM_005903.5	TIGIT	NM_173799.2	TNFSF4	NM_003326.2
PDGFB	NM_033016.2	PTPRC_all (common probe)	NM_080921.2	SOCS1	NM_003745.1	TIRAP	NM_148910.2	TNFSF8	NM_001244.3
PDGFRB	NM_002609.3	CD45R0	NM_080921.3	SOCS3	NM_003955.3	TLR1	NM_003263.3	TOLLIP	NM_019009.2
PECAM1	NM_000442.3	CD45RA	NM_002838.4	SPP1	NM_000582.2	TLR2	NM_003264.3	TP53	NM_000546.2
PIGR	NM_002644.2	CD45RB	ENST00000367367.1	SRC	NM_005417.3	TLR3	NM_003265.2	TRAF1	NM_005658.3
PLA2G2A	NM_000300.2	PYCARD	NM_013258.3	STAT1	NM_007315.2	TLR4	NM_138554.2	TRAF2	NM_021138.3
PLA2G2E	NM_014589.1	RAF1	NM_002880.2	STAT2	NM_005419.2	TLR5	NM_003268.3	TRAF3	NM_145725.1
PLAU	NM_002658.2	RAG1	NM_000448.2	STAT3	NM_139276.2	TLR7	NM_016562.3	TRAF4	NM_004295.2
PLAUR	NM_001005376.1	RAG2	NM_000536.3	STAT4	NM_003151.2	TLR8	NM_016610.2	TRAF5	NM_004619.3
PML	NM_002675.3	RARRES3	NM_004585.3	STAT5A	NM_003152.2	TLR9	NM_017442.2	TRAF6	NM_145803.1
POU2F2	NM_002698.2	RELA	NM_021975.2	STAT5B	NM_012448.3	TMEM173	NM_198282.1	TYK2	NM_003331.3
PPARG	NM_015869.3	RELB	NM_006509.2	STAT6	NM_003153.3	TNF	NM_000594.2	UBE2L3	NM_198157.1
PPBP	NM_002704.2	RORC	NM_001001523.1	SYK	NM_003177.3	TNFAIP3	NM_006290.2	VCAM1	NM_001078.3
PRDM1	NM_001198.3	RUNX1	NM_001754.4	TAGAP	NM_054114.3	TNFAIP6	NM_007115.2	VTN	NM_000638.3
PRF1	NM_005041.3	S100A8	NM_002964.3	TAL1	NM_003189.2	TNFRSF10C	NM_003841.3	XBP1	NM_005080.2
PRKCD	NM_006254.3	S100A9	NM_002965.2	TAP1	NM_000593.5	TNFRSF11A	NM_003839.2	XCL1	NM_002995.1
PSMB10	NM_002801.2	S1PR1	NM_001400.3	TAP2	NM_000544.3	TNFRSF13B	NM_012452.2	XCR1	NM_005283.2
PSMB5	NM_001130725.1	SELE	NM_000450.2	TAPBP	NM_003190.4	TNFRSF13C	NM_052945.3	ZAP70	NM_001079.3
PSMB7	NM_002799.2	SELL	NR_029467.1	TBK1	NM_013254.2	TNFRSF14	NM_003820.2	ZBTB16	NM_006006.4
PSMB8	NM_004159.4	SELPLG	NM_003006.3	TBX21	NM_013351.1	TNFRSF17	NM_001192.2	ZEB1	NM_001128128.1
PSMB9	NM_002800.4	SERPING1	NM_000062.2	TCF4	NM_003199.1	TNFRSF1B	NM_001066.2		

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Internal gene references	
Official Symbol	**Accession**
ABCF1	NM_001090.2
ALAS1	NM_000688.4
EEF1G	NM_001404.4
G6PD	NM_000402.2
GAPDH	NM_002046.3
GUSB	NM_000181.1
HPRT1	NM_000194.1
OAZ1	NM_004152.2
POLR1B	NM_019014.3
POLR2A	NM_000937.2
PPIA	NM_021130.2
SDHA	NM_004168.1
TBP	NM_001172085.1
TUBB	NM_178014.2
RPL19	NM_000981.3

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