Cape Peninsula University of Technology

MARKERS OF CHRONIC IMMUNE ACTIVATION AND T-CELL FUNCTION IN HYPERGLYCAEMIA

by

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DECLARATION

I, Tawanda Maurice Nyambuya, declare that the contents of this thesis represent my own unaided work, and that the thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

Signed **Date**

ABSTRACT

Introduction

Type 2 diabetes mellitus (T2DM) is a chronic inflammatory condition characterised by hyperglycaemia; continuous activation of T-lymphocytes and immune dysregulation. Although the exact mechanisms of these phenomena are not fully understood, there is strong evidence suggesting the involvement of T-cells in the chronic inflammatory environment which could predispose diabetics to infections and thrombotic events. The effect of hyperglycaemia on cells of the innate immune system in T2DM has been well described and implicated in the progression of the disorder and the development of its complications. However, studies investigating the adaptive immune response still remain scarce and controversial. Thus, investigating T-cells in hyperglycaemic conditions could provide further insight into the immune dysfunction observed in T2DM and assist in identifying pathways which could be targeted in the disease management and treatment. Therefore, this study aimed to investigate chronic immune activation by measuring the expression of T-cell activation markers in hyperglycaemia and compare the results to those in the normoglycaemic group.

Method

A total of 69 participants were recruited from Bellville South, Western Cape, South Africa. Thirty four (34) of the participants were hyperglycaemic, that is a 2hr oral glucose tolerance test (OGTT) value of $\geq$ 7.8mmol/l (140mg/dl) (included individuals with DM, IFG and/or IGT according to the WHO classification) and 35 were normoglycaemic. Standard flow cytometry methods were used to measure baseline levels of CD95, CD38, HLA-DR and PD-1 on T-cells in the hyperglycaemic group and the results were compared to those of a cohort of normoglycaemic individuals prior to incubating with glucose (pre glucose incubation). Thirty one (31) participants were randomly selected from the total sample population and sub grouped. Their T-cells were incubated with glucose before flow analysis (post glucose incubation). The expression of T-cell activation and exhaustion markers were correlated with glucose metabolism, routine biochemical and inflammation markers.

Results

Our results demonstrated no significant difference in the expression of T-cell activation antigens between the hyperglycaemic and normoglycaemic group pre incubation with glucose. Furthermore, the results showed no correlations between T-cell antigen expression and glucose metabolism. However, there was a significant correlation between ultra-sensitive C-reactive protein (CRP) and the expression of HLA-DR on $CD4^+$ T-cells and CD95 on all T-cell subsets. In the subgroup of participants whose T-cells were incubated with glucose, there was a significant difference in the mean florescence intensity (MFI) of HLA-DR on $CD4^+$

T-cells between the normoglycaemic and hyperglycaemic group at baseline. In addition, incubation with high concentrations of glucose significantly decreased the percentage of $CD4^+$ T-cells expressing CD95 in both groups.

Conclusion

We hypothesize that high concentrations of glucose suppress the immune system by decreasing the expression of $CD4^+$ T-cell activation markers which may lead to immune exhaustion.

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DEDICATION

To my beloved mother, Nespah Makoni.

Thank you for the unwavering support, unconditional love, patience, and sacrifices you made throughout my entire life. Last but not least, thank you for raising and moulding me into the man that I am today.

I love you!

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LIST OF ABBREVIATIONS

ADA	Association American Diabetes
AIDS	Acquired immunodeficiency syndrome
AICD	Activation induced cell death
ARV	Anti-retroviral drugs
AGEs	Advanced glycation end products
APCs	Antigen presenting cells
BMI	Body mass index
CD	Cluster Designation
CD3	T-cell back bone marker
CD4	T-helper cells
CD8	Cytotoxic T-cells
CD28	T-cell costimulatory molecule that binds to B7
CD38	cyclic ADP ribose hydrolase
CRP	C-reactive protein
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
CVDs	Cardiovascular diseases
DM	Diabetes Mellitus
Fas	CD95/Apo-1
FMO	Fluorescence minus one
HbA1c	Glycated haemoglobin
HDL	High-density lipoprotein
HIV	Human Immuno-deficiency virus
HLA-DR	Human Leucocyte Antigen- D related
HG	Hyperglycaemia (high blood glucose)
IDF	International Diabetes Federation
IFG	Impaired fasting glucose
IGT	Impaired glucose tolerance
IL	Interleukin

INF	Interferon
IRS-1	Insulin receptor substrate 1
ITAMs	Immuno-receptor tyrosine-based activation motifs
ITIM	Immuno-receptor tyrosine-based inhibiting motifs
ITSM	Immuno-receptor tyrosine-based switch motif
LDL	Low-density lipoprotein
MAP kinase	Mitogen-activated protein kinase
MFI	Mean fluorescence intensity
MCP-1	Monocyte chemotactic protein-1
MHC	Major-histocompatibility complex
MPO	Myeloperoxidase pathway
NF-kB	Nuclear factor-kappaB (transcriptional factor)
PAMPs	Pathogen associated molecular patterns
PD-1	Programmed cell death 1
PI3k/Akt	Phosphatidylinositol 3-kinase pathway
PKC	Protein kinase C
PMN	Polymorphonuclear leukocytes
RAGE	Receptor for AGEs
ROS	Reactive oxygen species
T_{eff}	Effector T-cells
T_{reg}	Regulatory T-cells
TCR	T-cell receptor
TLR	Toll-like receptors
TNF-α	Tumour necrotizing factor-α
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
WCC	White cell count
WHO	World Health Organisation

CHAPTER ONE

INTRODUCTION

1.1 Statement of research problem

Type 2 diabetes mellitus (T2DM) is a chronic inflammatory condition characterised by hyperglycaemia; continuous activation of T-lymphocytes and immune dysregulation (Pickup, 2004; Lontchi-Yimagou et al., 2013; Schietinger & Greenberg, 2014; Schietinger & Philip D, 2015). Although the exact mechanisms of these phenomena are not fully understood, chronic inflammation has been implicated in predisposing diabetics to infections, thrombosis and subsequently cardiovascular complications (Geerlings & Hoepelman, 1999; Erasmus et al., 2012; Matsha et al., 2012). Therefore, it is the intension of this study to investigate chronic immune activation and T-cell function in hyperglycaemia.

1.2 Background to the research problem

Studies investigating the role of cell-mediated immunity in diabetic patients and their susceptibility to infections are limited. Although results from various studies generally suggest impairment of immune function, the effects of hyperglycaemia on peripheral T-lymphocytes remain scarce (Geerlings & Hoepelman, 1999; Stegenga et al., 2008; Wu et al., 2011). In addition, the effects of glucotoxicity on peripheral T-cell function have not been reported in the targeted South African mixed ancestry population (Erasmus et al., 2012; Matsha et al., 2012). In this study, T-cell activation state and effects of high glucose concentrations on T-cell antigen expression will be investigated.

1.3 Research aim

- To investigate markers of chronic immune activation and T-cell function in hyperglycaemia.

1.4 Research objectives

- To measure the expression of activation and exhaustion markers on T-cells in hyperglycaemic and normoglycaemic individuals.
- To investigate the effects of varying concentrations of glucose on T-cell antigen expression.
- To correlate the expression of T-cell activation and exhaustion markers with glucose metabolism, routine biochemical markers and inflammation.

CHAPTER TWO
LITERATURE REVIEW

2. Introduction

The global prevalence of diabetes mellitus (DM) has significantly increased from 4.7% in 1980 to 8.5% in 2014 (WHO, 2016).This increase has been attributed to urbanisation and modernisation of the developing world, particularly in Sub-Saharan Africa (Mbanya et al., 2010; Matsha et al., 2012; Renzaho, 2015). Estimates from 2009 by the International Diabetes Federation (IDF) suggested that the prevalence of DM in Sub-Saharan Africa will increase from 4.8% to 5.3% by the year 2035 (IDF, 2009). Despite the high rate of undiagnosed DM cases in Sub-Saharan Africa (Mbanya et al., 2010), a total of 19.8 million cases of overt diabetes and a prevalence of about 7.1% has been reported in the year 2013 and 2014 respectively (IDF, 2013; WHO, 2016).

In South Africa, a total of 3.5 million people, about 8% of the total population, is diabetic (Molleutze & Levitt, 2006; IDF, 2013). A previous report indicated that DM is the fifth highest cause of natural deaths in South Africa and number one in the Western Cape province (Statistics South Africa, 2013). Furthermore, studies have shown that the mixed ancestral population of South Africa has the second highest prevalence of DM after the Indian population (Levitt et al., 1999; Motala et al., 2003).

Type 2 Diabetes Mellitus (T2DM), is an inflammatory condition associated with increased risk of cardiovascular diseases (CVDs) and thrombosis (Matsha et al., 2012; Vazzana et al., 2012). The chronic hyperglycaemia observed in this disorder induces a state of low grade systemic inflammation responsible for the immune activation that precedes insulin resistance in the pathogenesis of the disease (Dokken, 2008; Lontchi-Yimagou et al., 2013). This suggests that there is a strong relationship between hyperglycaemia, inflammation, immune activation and CVDs. Consequently, the hyperglycaemic state in uncontrolled T2DM seems to induce chronic activation, exhaustion and ultimately the dysfunction of T-lymphocytes (Brod, 2000; McKinney et al., 2007; Wang et al., 2014).

The aim of this review is to examine the process of inflammation, immune activation and exhaustion and how these processes are linked to the pathogenesis of T2DM. Literature was searched on Google scholar and PubMed using terms “Immune system”, “Inflammation”, “T-cell function”, “T2DM”, “T-cell activation and exhaustion”, “Programmed cell death (apoptosis)” and “T-cell dysfunction in DM and other inflammatory conditions’’.

2.1 Definition and classification of Diabetes Mellitus

Diabetes Mellitus is a group of disorders characterised by chronic hyperglycaemia due to defects in insulin secretion, action or both (ADA, 2009). Individuals with uncontrolled hyperglycaemia often present with polyuria, polydipsia and unexplained weight loss which is sometimes coupled with polyphagia and blurred vision (ADA, 2009; Kitabchi et al., 2009). The clinical criteria for the diagnosis of DM includes (Table 2.1), a fasting plasma glucose (FPG) of ≥ 7.0 mmol/l (126mg/dl) or 2-hour plasma glucose of ≥ 11.1mmol/l (200mg/dl) (WHO, 2006)

Table 2.1: Criteria for the diagnosis of DM (compiled by author, based on work WHO, 2006)

1. FPG $\geq$ 7.0mmol/l (126mg/dl). Fasting is defined as no caloric intake for at least 8 hours.

   Or

2. A 2-hour plasma glucose of ≥ 11.1mmol/l (200mg/dl) during an oral glucose tolerance test (OGTT).

2.1.2 Pre diabetes

The World Health Organisation (WHO) identified an intermediate group of individuals whose glucose levels do not meet the criteria to be diagnosed as diabetics, yet their levels are higher than those considered to be normal (Harris et al., 1997). These individuals have been referred to as pre-diabetics and can be identified as having either impaired fasting glucose (IFG) or impaired glucose tolerance (IGT) (Alberti, 2007; Grundy, 2012). Global statistics have indicated that an estimated 316 million people are pre-diabetic and have a high risk of developing T2DM (Grundy, 2012; IDF, 2013). A previous report indicated that the prevalence of pre-diabetics in the Cape Town mixed ancestry population was 4.3% (Erasmus et al., 2012).

The use of the term “pre-diabetes” has been debated over the years and the WHO discouraged its use during the 1980s as it was reported that many of these individuals did not proceed to clinical T2DM (Grundy, 2012). The term, pre-diabetes was however re-introduced in 2005 by the American Diabetes Association (ADA) to describe individuals with impaired glucose tolerance (IGT) of 7.8–11.0mmol/l (140–199mg/dl), impaired fasting glucose of 6.1–7.0 mmol/l(110–125mg/dl) and most recently HbA1c (5.7–6.4%) (ADA, 2009). The WHO in 2006, introduced the term “intermediate hyperglycaemia” to describe IGT and IFG since these individuals can either revert back to a normoglycaemic state or proceed to clinical T2DM (Table 2.2) (WHO, 2006).

Table 2.2: Criteria for the diagnosis of intermediate hyperglycaemia (compiled by author, based on work WHO, 2009).

1. Impaired fasting glucose (IFG) of 6.1–6.9mmol/l (110–125mg/dl)

Or

2. Impaired glucose tolerance (IGT):
    - Fasting plasma glucose of < 7.0mmol/l (125mg/dl)

and

    - A 2hr plasma glucose of ≥ 7.8mmol/l (140mg/dl) and < 11.0mmol/l (200mg/dl)

2.1.2 Type 1 DM

Type 1 diabetes mellitus (T1DM) is an autoimmune disorder characterised by β-cell destruction induced by auto antibodies directed against the insulin secreting pancreatic β-cell (Cnop et al., 2014). Although strong evidence of immune dysfunction has been reported in the pathogenesis of T1DM (Al Homsi & Lukic, 1992.; Ozougwu, 2013), it is beyond the scope of this study.

2.1.3 Type 2 DM

Type 2 diabetes mellitus (T2DM) is characterised by a combination of insulin resistance and an inadequate compensatory insulin secretory response with relative insulin deficiency (Kahn, 2003; ADA, 2009). This type is usually diagnosed in older patients with predisposing risk factors such as obesity, hypertension and hypercholesterolaemia (Kohei, 2010). Type 2 DM accounts for over 90% of diabetes cases in Sub-Saharan Africa (Levitt, 2008) and in South Africa, it has a prevalence of 8 % (IDF, 2013). Provincial statistics have shown that the Western Province has the highest prevalence of DM with an incidence of 8.1% (Erasmus et al., 2012).

In normal glucose metabolism, a narrow plasma glucose concentration range is maintained despite wide fluctuations in supply and demand. This is achieved by regulated interactions between tissue sensitivity to insulin (especially in liver) and insulin secretion (ADA, 2009; Ozougwu, 2013). However, in T2DM, a combination of genetic factors related to impaired insulin resistance (especially by the liver cells), insulin secretion (dysfunctional pancreatic β cells) as well as environmental factors such as obesity, lack of exercise and stress (Fig 2.1) are implicated in the disruption of the plasma glucose balance and the development of T2DM (Kohei, 2010; Ozougwu, 2013). Biochemically, T2DM may present with a high white cell count, pro-inflammatory cytokines and chemokines, coagulation factors (such as fibrinogen

and plasminogen activator inhibitor 1) and acute-phase proteins (King, 2008; Esser et al., 2014; Hu et al., 2015).

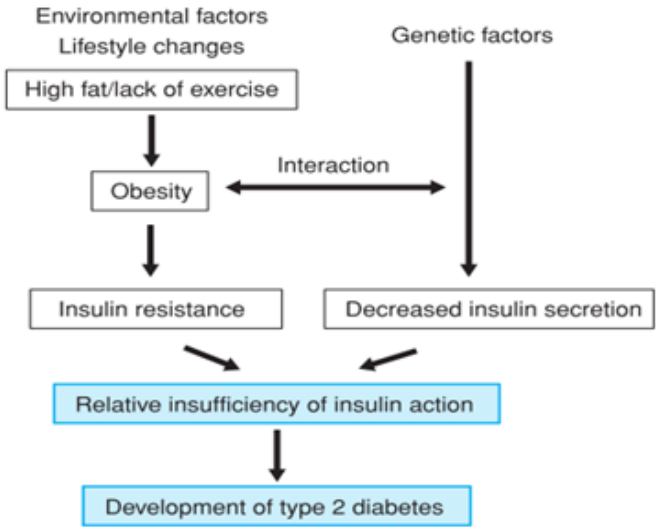

Figure 2.1: Etiology and pathogenesis of T2DM (adapted from Kohei, 2010). Type 2 DM is a multifactorial disease involving a combination of genetic and life style environmental factors related to impaired insulin secretion and resistance.

Previous studies have demonstrated a link between T2DM, obesity and abdominal adipose tissue. The adipose tissue adipocytes become hypertrophic in an effort to accommodate excess triglyceride storage and eventually undergo apoptotic cell death and adipokine secretion (Chng et al., 2015; Ip et al., 2015). This process attracts cells of the innate immune system such as macrophages and monocytes which trigger the production of pro-inflammatory cytokines and initiate an adaptive immune response (Chng et al., 2015). Notably, the secretion of these pro-inflammatory biomarkers by the adipocytes correlates with the prevalence and incidence of complications associated with T2DM such as thrombosis and CVDs (Lontchi-Yimagou et al., 2013). Although the abdominal adipose tissue has been identified as a source of pro-inflammatory cytokines and chemokines such as IL-6, TNF-α and monocyte chemoattractant protein-1 (Hotamisligil et al., 1995; Fried et al., 1998; Catalán et al., 2007), the bulk of these pro-inflammatory biomarkers are secreted by circulating and tissue-associated immune cells (King, 2008; Hameed et al., 2015).

2.2 The Immune system

The immune system is an organization of cells and molecules with specialized roles and has the ability to distinguish self from non-self in defending the body against infection (Medzhitov, 2007). Two general forms of the immune system exist, namely innate immunity and adaptive immunity (Parkin & Cohen, 2001).

2.2.1 Innate immunity

The innate immunity is classically known to be antigen non-specific and lack immunological memory. This includes physical barriers, phagocytic cells (neutrophils, monocytes and macrophages), cells that release inflammatory mediators, basophils, mast cells, eosinophils and natural killer cells (Delves & Roitt, 2000). The innate response also includes soluble proteins such as complement, cytokines and chemokines that are either naturally present in biological fluids or are released from cells as they are activated (Chaplin, 2010).

Macrophages, possess receptors for carbohydrates such as mannose, which are not normally exposed on vertebrate cells (Delves & Roitt, 2000). During phagocytosis, the engulfed microbes are subjected to neutralising toxic intracellular molecules, which includes superoxide anion, hydroxyl radicals, antimicrobial cationic proteins and lysozyme (Delves & Roitt, 2000). In addition, these phagocytes are responsible for the clearance of apoptotic and/or necrotic cells and subsequently release pro-inflammatory mediators (Savill, 1997).

The innate immune system activates the adaptive immune response by presenting antigens to T-lymphocytes (Fig 2.2). The dendritic cells are activated via the recognition of distinctive pathogen-associated molecular patterns (PAMPs) which are present on the surface of a micro-organism (Medzhitov, 2008). This leads to the presentation of antigen by antigen presenting cells (APCs) via major-histocompatibility-complex (MHC) molecules and the activation of naive T cells (Kronin et al., 2001; Medzhitov, 2007). Furthermore, the activation of dendritic cells enhances the surface expression of B7 (CD80/86), a co-stimulatory molecule which is essential for providing the second co-stimulatory signal in addition to the antigenic signal, for successful T-cell activation (Harris & Ronchese, 1999). Antigen presenting cells present the MHC and antigen complex to the T-cell receptors (TCR) on $CD28^{+}$ naïve T-cells which become activated and initiate the adaptive immune response (Delves & Roitt, 2000).

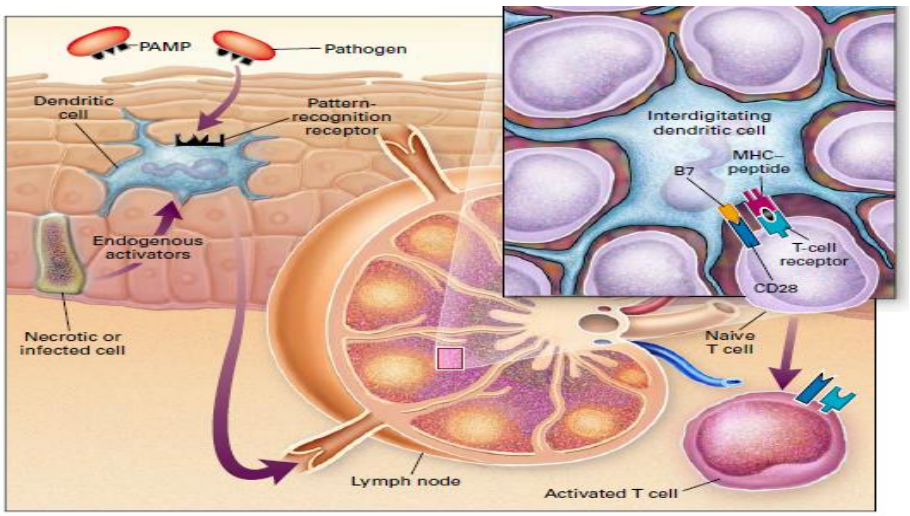

Figure 2.2: T cell activation by dendritic cell (adapted from Delves & Roitt, 2000). The pathogen-associated molecular patterns (PAMPs) receptors on antigen presenting cells (APCs) enable them to distinguish between potentially harmful foreign microorganisms and self-antigens. Innate immunity APCs such as dendritic cells and macrophages present foreign antigens peptide on major-histocompatibility-complex (MHC) to T-cell receptors on $CD28^{+}$ naïve T-cells thereby activating acquired immunity. This activation also triggers dendritic cells to upregulate expression of B7 costimulatory molecules, essential for providing the second stimulatory signal for successful activation of T-cells.

2.2.2 Adaptive immunity

Adaptive immunity is tightly regulated by APCs (Delves & Roitt, 2000). The T and B lymphocytes enable the immune system to distinguish between non-self and self-antigens and initiate pathogenic-specific immunologic effector pathways (Bonilla & Oettgen, 2010). Once the antigen is eliminated, immunologic memory cells are developed and immunological homeostasis is maintained (Bonilla & Oettgen, 2010; Warrington et al., 2011).

Antibody-producing B-lymphocytes and T-lymphocytes develop in the primary lymphoid organs, the bone marrow and thymus respectively. Once fully developed, the lymphocytes migrate to secondary lymphoid organs for specific antigen capturing (Bonilla & Oettgen, 2010). The proliferation of naïve lymphocytes after encountering antigen leads to priming, activation and differentiation (Fig 2.3) into both T and B effector cells and memory cells (Chaplin, 2010). The memory cells enable an effective secondary immune response to be mounted after a subsequent encounter with the same antigen (Delves & Roitt, 2000). Activated T-cells secrete pro-inflammatory mediators that initiate an inflammatory response.

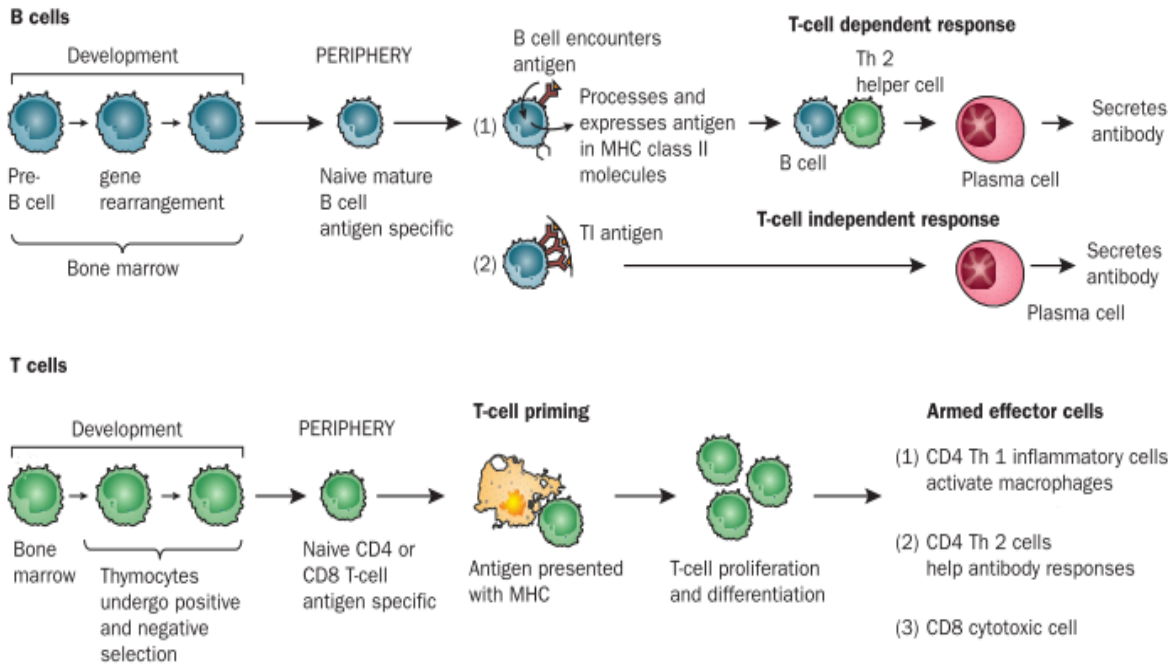

Figure 2.3: T and B cells in adaptive immunity (adapted from Parkin & Cohen, 2001). The hall mark of adaptive immunity is the use of antigen-specific receptors on T and B-cells to drive targeted effector responses. The initial presentation of antigens to naïve T and B-cell by antigen presenting cells (APCs) leads to cell priming, activation, and differentiation. The activated T and B-cells then migrate from the lymphoid tissue to the inflammation site and release of plasma antibodies into blood and tissue fluid respectively.

2.3 Inflammation

Inflammation can be divided into two phases, namely the acute phase and the chronic phase which is characterised primarily by its persistence and lack of clear resolution (Ward, 2010; Maskrey et al., 2011). During inflammation, vascular permeability and blood flow is increased. As a result leukocytes, platelets and plasma constituents migrate from the blood vessels into the injured tissue (Ley et al., 2007; Maskrey et al., 2011; Wu & Chen, 2014). Mediators of inflammation involve both humoral and cellular factors as well as molecular factors (Cicala & Cirino, 1998; Maskrey et al., 2011). Acute inflammation subsides shortly after neutralization of the infection is achieved and repair of the damaged tissue is initiated (Maskrey et al., 2011). However, in T2DM, the inflammation process is unregulated and chronic, leading to chronic immune activation, exhaustion and ultimate immune dysfunction (Pickup, 2004; Schietinger & Greenberg, 2014).

2.3.1 Acute inflammation

Acute inflammation is a primary component of the innate immune system that may be triggered by tissue damage or pathogens. In an acute inflammatory response, systemic reactions take place that result in increased concentration of acute-phase reactants via leakage of water, salt, and proteins from the vascular compartment and activation of endothelial cells (Fig 2.4) (Pickup, 2004). The opening of endothelial cells tight junction allows activated neutrophils to move up the chemotactic gradient to the site of infection for phagocytosis (Delves & Roitt, 2000).

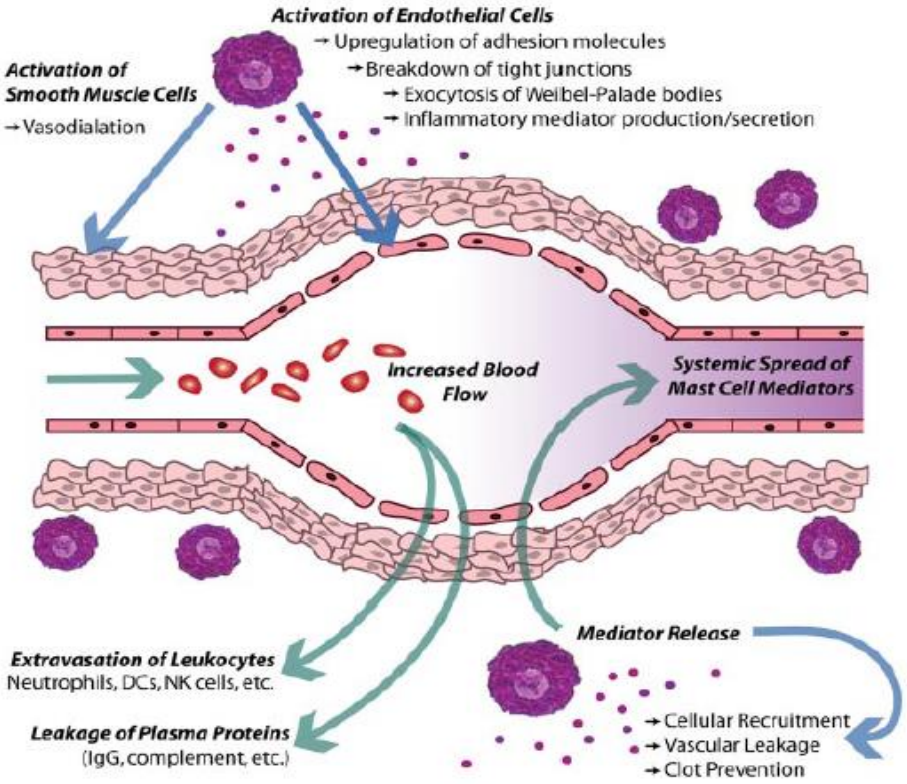

Figure 2.4: The acute-phase inflammatory response (adapted from Kunder et al., 2011). During this process, the initial step involves vascular dilation, increased blood flow (causing erythema and warmth) and activation of endothelial cells. Concurrently, there is a structural change that causes increased vascular permeability prompting extravasation of plasma proteins and leukocytes causing oedema. The final step involves emigration of leukocytes from the microcirculation, their accumulation in the focus of injury, and their activation to eliminate the offending agent.

The adhesive interactions between platelets, leukocytes and the vascular endothelium leads to the recruitment of leukocytes and the activation of tissue macrophages and platelets (Ward, 2010). This is followed by the activation of complement and clotting systems with the subsequent release of proteases and oxidants from phagocytic cells (Pickup, 2004; Ward, 2010). The *in vivo* hallmark of this response is the adhesion of neutrophils to the vascular endothelium (margination), a process which initiates platelet activation and coagulation (Ley et al., 2007).

When an acute inflammatory response is triggered, complement is activated which triggers the degranulation of mast cells. This results in the release of histamines responsible for contraction of smooth muscles and an increase in vascular permeability (Delves & Roitt, 2000; Kunder et al., 2011). This results in the activation of endothelial cells and the up-regulation of endothelial adhesion molecules and tissue factor (TF) (Rivera-Nieves et al., 2008; Ward, 2010). The rolling of neutrophils along the vessel wall is halted by the expression of adhesion molecules which bind to selectins and integrins expressed on the cell surface of activated neutrophils (Delves & Roitt, 2000). The leukocyte-endothelial interactions initiate the release of pro-inflammatory cytokines and chemokines at the site of inflammation (Wahi et al., 1996). These pro-inflammatory molecules chemotactically attract and activate neutrophils prior to their transmigration into the extravascular matrix (Wahi et al., 1996; Rivera-Nieves et al., 2008; Ward, 2010).

Once resolution is achieved, natural occurring anti-inflammatory factors are released to downregulate the inflammatory process before causing tissue damage (Pickup, 2004; Ward, 2010; Wu & Chen, 2014). These anti-inflammatory factors include cytokines (Interleukin (IL) 4, IL-10, IL 12), protease inhibitors; antioxidant enzymes; lipoxins; kinases and transcriptional factors (Ward, 2010). Despite the acute phase inflammatory response being tightly regulated and short lived, in T2DM, it is unregulated and chronic (Pickup, 2004).

2.3.2 Chronic-inflammation

The chronic inflammatory phase is a prolonged and dysregulated immune response that involves active inflammation, tissue destruction and attempts at tissue repair (Ward, 2010; Murakami & Hirano, 2012). This is due to a persistent stimulus and failure of the acute-phase response to provide any resolution (Weiss, 2008). In the chronic inflammatory response, the neutrophils are replaced by macrophages, plasma cells and T cells (Weiss, 2008). Upon failure of the acute inflammatory process and macrophages to phagocytose pathogens, tertiary lymphoid tissues get involved and in certain diseases, granulomas are formed (Medzhitov, 2008; Weiss, 2008).

Therefore, chronic inflammation is characterised by the persistent activation of T-cells, which is the hallmark of chronic inflammatory conditions such as T2DM and CVDs (Levine, 2005; Medzhitov, 2008). Thus, T-cells play an important role in the pathogenesis and progression of T2DM as a chronic inflammatory condition.

2.4 T-cell maturation and tolerance

The maturation of bone marrow stem cells consist of three processes namely, proliferation of immature cells, expression of receptor chains by VDJ gene recombination (Mikkola & Orkin, 2006; Chaplin, 2010). During the VDJ recombination process, T-cells which fail to express

functional receptors do not receive survival signals and undergo apoptosis (Goldrath & Bevan, 1999). After leaving the bone marrow, immature T-cells migrate to the thymus where they become thymocytes and are educated to be self-tolerant prior to their departure to the secondary lymphoid organs (Delves & Roitt, 2000).

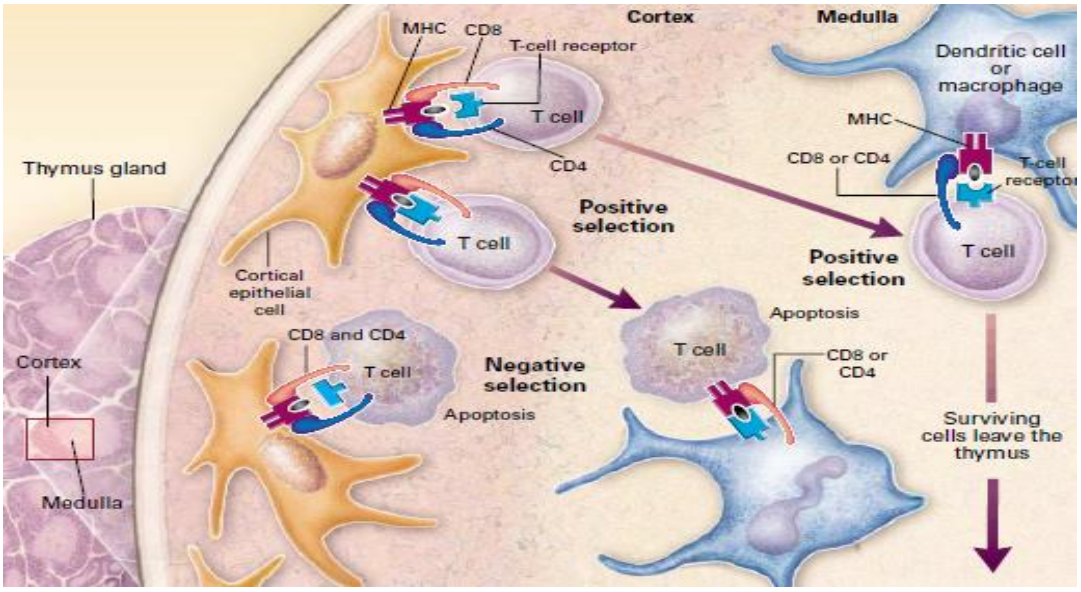

Figure 2.5: T-cell receptor-major-histocompatibility complex (TCR–MHC) interactions in positive and negative selection of thymocytes (adapted from Delves & Roitt, 2000). The TCR-MHC affinity interactions facilitate maturation, survival, proliferation or apoptotic cell death (ACD) of thymocytes. Thymocytes that express TCR with high affinity to MHC complex either trigger programmed cell death or differentiate into Treg via the negative selection. Those with intermediate affinity undergo positive selection and differentiate into single-positive effector $CD4^+$ or $CD8^+$ and migrate to secondary lymphoid organs. Thymocytes that show no sign of affinity die by neglect.

T-cell selection is determined by their binding affinity to MHC molecules (Fig 2.5) (Goldrath & Bevan, 1999; Camara et al., 2012). When T-lymphocytes are exposed to antigens they may become activated, proliferate and differentiate into effector cells leading to an immune response (Camara et al., 2012). When T-cells undergo apoptosis or are functionally inactive during the selection process, they are said to be tolerant. Immunological tolerance of self-reactive T-cells in the thymus and peripheral lymphoid organs is termed central tolerance and peripheral tolerance, respectively (Romagnani, 2006). The phenomenon of immunological tolerance is important in several aspects as its failure may result in autoimmunity and immune dysfunction.

2.4.1 Central tolerance

The thymus mediates the selection process by providing a suitable microenvironment with a specific combination of stromal cells, cytokines and chemokines to generate functional T-cells (Goldrath & Bevan, 1999; Luckheeram et al., 2012). When immature double negative ($CD4^-CD8^-$) T-cells migrate from the bone marrow into the thymus, most of them express an αβ T-cell receptor (Delves & Roitt, 2000; Parkin & Cohen, 2001). The expression of the αβ TCR heterodimers and upregulation of $CD4^+CD8^+$ (double positive) thymocytes allows cells

with a functional TCR to be positively selected by MHC in the thymic cortex (Metzger & Anderson, 2012). Thymocytes with intermediate affinity to a particular self-MHC haplotype survive and mature to either $CD4^+$ or $CD8^+$ single positive T-cells (Goldrath & Bevan, 1999; Camara et al., 2012; Luckheeram et al., 2012; Metzger & Anderson, 2012).

T-cells that have passed the positive selection process migrate to the thymic medulla where they undergo negative selection (Goldrath & Bevan, 1999). This process prevents the maturation of T-cells with strong affinity to the MHC-self peptide complexes present on the surface of medullary epithelial cells (MEC) (Romagnani, 2006). Although the number of self-antigens present within the thymus are limited, the promiscous gene expression (pGE) regulated by the autoimmune regulator (AIRE) gene, allows the MEC to present a vast range of self-antigens.(Romagnani, 2006; Metzger & Anderson, 2012). Thymocytes expressing a TCR with very high affinity or no affinity to self-antigens expressed on the MHC complex undergo negative selection by either apoptotic cell death (ACD) or differentiation into natural regulatory T-cells (Treg) (Goldrath & Bevan, 1999; Camara et al., 2012). Only 3% of thymocytes in the thymus survives both positive and negative selection processes (Romagnani, 2006).

2.4.2 Peripheral tolerance

Despite the efficient central tolerance mechanisms, not all self-reactive T-cells are eliminated and may migrate to secondary lymphoid organs where they pose a risk of mounting an autoimmune response. Therefore, a secondary mechanism termed peripheral tolerance takes place to prevent the activation of autoreactive T-cells. This involves three key mechanisms that include; anergy, deletion and immune suppression.

2.4.2.1 Anergy

Both the TCR and CD28 co-stimulatory molecule signals are required for successful activation of T-cells (Fig 2.9) (Harber et al., 2000). In the absence of a co-stimulatory signal, a hypo-responsive state of T-cells termed anergy is induced. This occurs despite active TCR signalling and IL-2 expression (Xing & Hogquist, 2012). Therefore, albeit co-stimulatory signals are essential for T-cell activation, they can also provide a negative signal that may inhibit the activation process, induce tolerance and prevent autoimmunity (Fig 2.6) (Liechtenstein et al., 2012; Xing & Hogquist, 2012). The programmed cell death 1 (PD-1) receptor and its ligands (PD-L1 and PD-L2) and the cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) pathways also play an important role in controlling the immune response by inducing T-cell anergy (Liechtenstein et al., 2012).

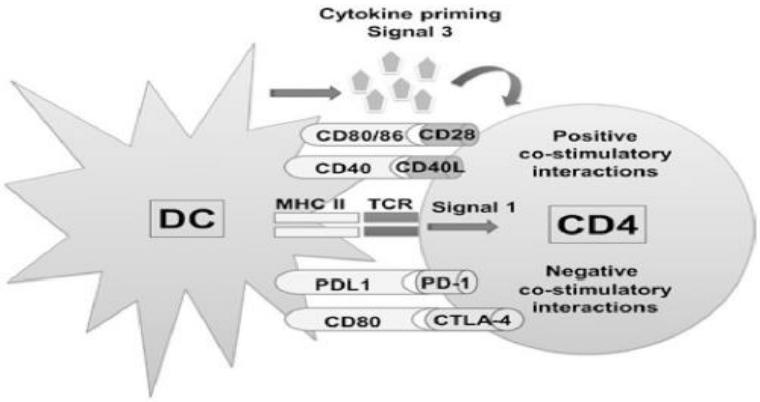

Figure 2.6: Programmed cell death-1 and its ligands (PD1-L) pathway and cytotoxic T-lymphocyte-associated 4 (CTLA-4) in peripheral anergy (adapted from Liechtenstein et al., 2012) .The TCR-MHC and CD28-CD80/86; CD40-CD40L ligation provides initial and co-stimulatory signals respectively for successful activation of T-cells and subsequent release of cytokines. The PD1-L pathway and the CD80-CTLA-4 provide negative co-stimulatory signals which inhibit T cell activation resulting in anergy. T-cell anergy is essential in inducing T-cell peripheral tolerance that would otherwise result in autoimmunity.

The PD-1 receptor is a member of the CD28 superfamily that delivers an inhibitory signal upon ligand binding (Jin et al., 2011) The co-stimulatory response of the PD-1-L pathway occurs during antigen presentation to naïve T-cells by APCs or during cytotoxic T-cell attack (Liechtenstein et al., 2012). Upon activation, T-cells up-regulate PD-1 interactions with PD1-L resulting in co-stimulation and ligand-induced TCR down-modulation (Liechtenstein et al., 2012). In turn, TCR down-modulation terminates the intracellular signal transduction pathway by the recruitment of phosphatase (SHIP-1 and SHIP-2) (Liechtenstein et al., 2012; Xing & Hogquist, 2012). SHIP-1 dephosphorylates proximal signalling molecules and inhibits the PI3K pathway which is essential for T-cell activation (Liechtenstein et al., 2012; Xing & Hogquist, 2012; Jin et al., 2011).

In addition, T-cell activation also induces the up-regulation of CTLA-4, which competes with the CD28 co-stimulatory molecule for binding to B7 (CD80/86) (McCoy & Le Gros, 1999). Once the CTLA-4 binds to B7, it delivers an inhibitory signal that inhibits T-cell activation (Romagnani, 2006). Therefore, T-cells do not necessarily die but persist in the body as functionally inactive effector cells termed anergy.

2.4.2.2 Deletion

This mechanism of peripheral tolerance is based on ACD which is mediated by Fas/APO-1 (CD95) and other death receptors on the surface of the T-cell (Fig 2.7) (Askenasy et al., 2005). Chronic activation of T-cells induces the upregulation of Fas and Fas ligand (FasL) which triggers apoptosis (Askenasy et al., 2005). The Fas/FasL pathway activates a cascade of caspase enzymes that results in activation-induced cell death (AICD) (Van Parijs et al., 1996; Romagnani, 2006; Askenasy et al., 2005). Consequently, dysregulation of the Fas/FasL pathway may result in lymphoproliferative disease, autoimmune diseases as well

as dysfunctional T-cell mediated adaptive immune response (Geerlings & Hoepelman, 1999; Schietinger & Philip D, 2015)

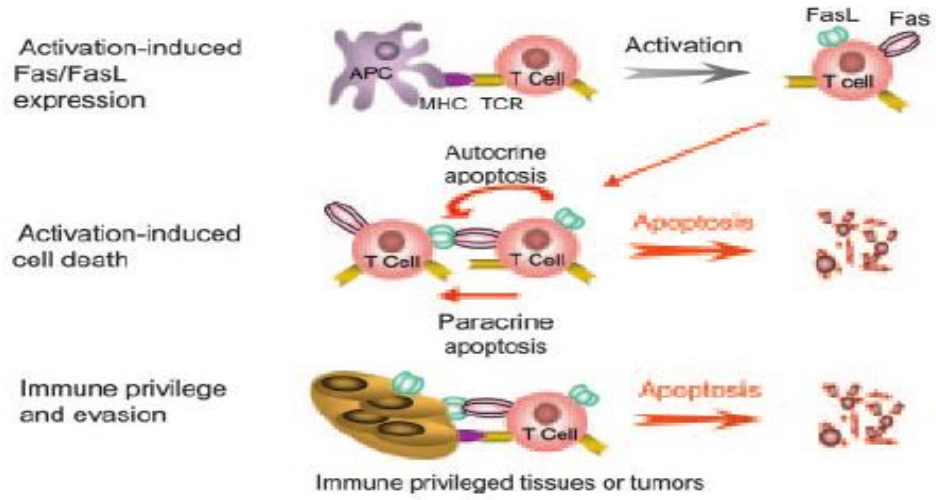

Figure 2.7: Fas mediated apoptosis in peripheral tolerance (adapted from Askenasy et al., 2005). Activation of T-cells results in the upregulation of Fas and Fas ligand expression. Chronic T-cell activation may trigger the Fas/FasL mediated autocrine and paracrine apoptosis termed activation-induced cell death (AICD). The AICD results in the elimination of self- reactive T-cells.

2.4.2.3 Immune suppression by natural T regulatory cells

T regulatory (T_{reg}) cells are thymus derived T-cell subsets that are $CD4^+$ $CD25^+$ and express FOXP3, a transcription factor that plays an important role in their development and function (Alpdogan & Van den Brink, 2012). They do not respond to TCR stimulation and possess the ability to suppress the proliferation of other T-cells (Fritzsching et al., 2009; Alpdogan & Van den Brink, 2012). The T_{reg} cells are essential in inducing immunologic self-tolerance following antigen priming in a relevant cytokine environment (Sakaguchi et al., 2006) (Sakaguchi et al., 2006; Fritzsching et al., 2009; Alpdogan & Van den Brink, 2012).

The survival and function of T_{reg} is also dependent on IL-2, a pro-inflammatory cytokine which promotes the differentiation of T-cells into T_{reg} cells (Wan & Flavell, 2007) and therefore express CD25 (IL-2R) (Alpdogan, 2013). The expression of FOXP3 may be upregulated on naïve T-cells during chronic activation in the presence of transforming growth factor (TGF)- β (Chen et al., 2003). In addition, the secretion of inhibitory cytokines such as TGF-β and IL-10 by T_{reg} and their direct interaction with B7 (CD80/CD86) via the CTLA-4 molecule results in the suppression of effector T-cells (T_{eff}) (Fig 2.8) (Alpdogan, 2013).

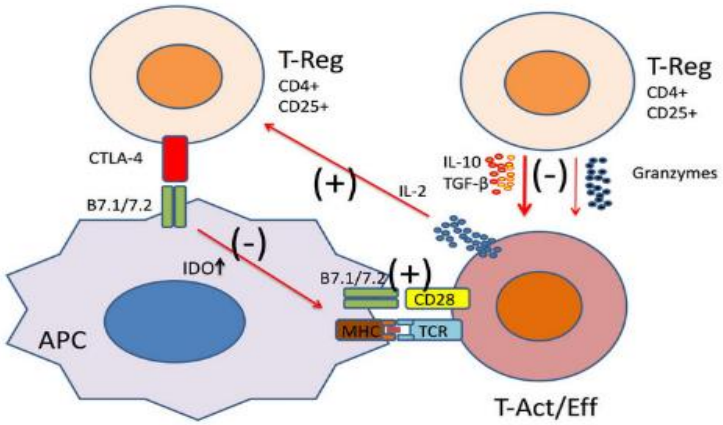

Figure 2.8: Regulatory T-cells (T_{reg}) inhibiting T-cell effector (T_{eff}) function (adapted from Alpdogan, 2013). T_{reg} suppress T_{eff} cells by secreting inhibitory cytokines such as transforming growth factor (TGF)-β and interleukin-10 (IL10) which decreases T-cell activity by promoting the expansion of T_{reg} cells and inducing either anergy or apoptosis respectively. In addition, the interaction of T_{reg} cells expressing CTLA-4 with antigen presenting cells (APCs) activate the indoleamine 2,3 dioxygenase (IDO) enzyme which initiate the production of T-cell function inhibitory molecules such as kynurenine. Interluekin-2 (IL-2) is essential for the survival and function of T_{reg} cells.

2.5 Markers of T-cell immune activation

Numerous cell surface antigens have been utilised to investigate levels of T-cell activation and include CD25, CD69, CD70, CD38 and HLA-DR (Deeks et al., 2004; Eggena et al., 2005; Saber et al., 2007; Dieterlen et al., 2014). For this study, CD38 and HLA-DR were the markers of choice as they have been widely accepted as robust markers of T-cell activation (Hertoghe et al., 2000; Feuth et al., 2013; Funderburg et al., 2013; B. Nkambule et al., 2015).

2.5.1 Human Leukocyte Antigen-D Related (HLA-DR)

The Human Leukocyte Antigen-D antigen (HLA-DR) is a well-known marker of immune activation due to its increased expression on activated leukocytes (Hertoghe et al., 2000; Feuth et al., 2013). It is a MHC class II cell surface receptor encoded by the human leukocyte antigen complex that binds with the TCR in T-cell activation (Chung et al., 1994). The expression of HLA-DR antigens is upregulated on activated APCs (B-cells, monocytes/macrophages and dendritic cells) in order to present antigen to $CD4^+$ T-cells (Chaplin, 2010). This stimulates an adaptive immune response resulting in pro-inflammatory cytokine production (Salgado et al., 2002; Ueno et al., 2007). Therefore, since the expression of HLA-DR is normally absent on resting T-cells and subsequently up-regulated on activated T-cells, it is a robust activation marker that indicates active proliferation of immune cells as well as production of pro-inflammatory cytokines (Viallard et al., 2001; Salgado et al., 2002). The combinational use of CD38 and HLA-DR as activation markers has been used in several studies of inflammatory conditions (Hunt et al., 2008; Feuth et al., 2013; Funderburg et al., 2013; Smith et al., 2013).

2.5.2 Cyclic ADP ribose hydrolase (CD38)

CD38 is an immunoregulator ectoenzyme which catalyses the synthesis and hydrolysis of cyclic adenosine diphosphate–ribose (cADPR) which is responsible for both the synthesis and degradation of several Ca^{2+} depended second messengers (Pavon et al., 2013). In addition, CD38 catalyses the transglycosylation reaction by converting nicotinamide adenine dinucleotide (NAD) into another important intracellular transduction signalling molecule, nicotinic acid adenine dinucleotide phosphate (NAADP), hence it is referred to as a bi-functional ectoenzyme (Hamblin, 2003; Pavon et al., 2013).

Normally, early bone marrow T-cell precursors, thymocytes and peripheral T-cells have approximately 6%, 90% and 8% expression of CD38 on their cell surfaces respectively (Malavasi et al., 1992). The expression of CD38 on the cell surface is highly dependent on the state of cellular activation and differentiation (Mehta et al., 1996; Pavon et al., 2013). In activated T-cells, the expression of CD38 is upregulated hence it is useful in the study of T-cell activation (Funderburg et al., 2013; Smith et al., 2013; Nkambule et al., 2015). Interestingly, high glucose-utilising tissue particularly the pancreas, naturally express high levels of CD38, which is believed to play a pivotal role in glucose-induced insulin secretion from the islets cells (Mehta et al., 1996).

2.5.2.1 CD38 signalling pathway

The cyclic ADP ribose hydrolase pathway is responsible for the synthesis of two second messengers responsible for mobilising Ca^{2+} stores namely cADPR and NAADP (Fig 2.9). The main ADP-ribosyl cyclase in mammals is CD38 (Wei et al., 2014). The cADPR acts on the ryanodine receptors (RyR) inside the endoplasmic reticulum and NAADP mobilises the endolysosomes via the two-pore channels (TPC) (Lee, 2012). The endogenous Ca^{2+} mobilising nucleotide, cADPR which is responsible for calcium release or influx is synthesised from NAD in the presence of CD38 (Wei et al., 2014) and NAADP is derived from NAD via the catalysis of the CD38 enzyme (Lee, 2012; Wei et al., 2014). The CD38 pathway modulates various processes including insulin secretions (Mehta et al., 1996), cardiac regulation (Reyes et al., 2015) and inflammation (Deshpande et al., 2004; Sathish et al., 2014). Since the discovery of CD38 as a lymphocyte antigen, its pathway has been shown to play a crucial role in inflammatory processes (Horenstein et al., 2013).

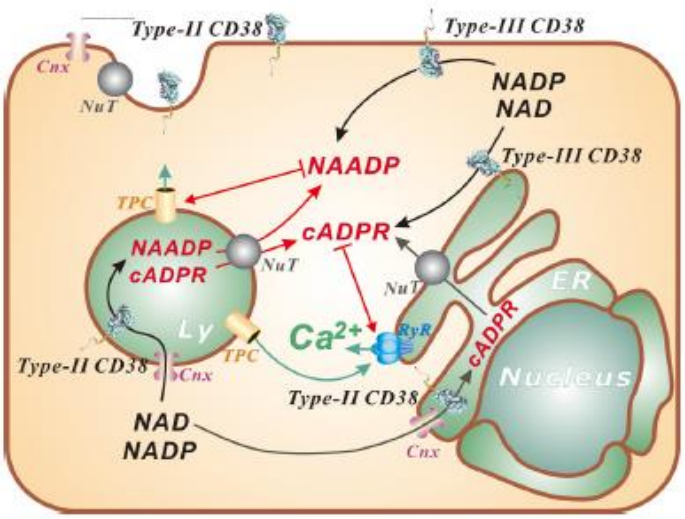

Figure 2.9: CD38 signalling pathway (adapted from Lee, 2012). The cADPR synthesised from NAD via the action CD38 acts on the ryanodine receptors (RyR) inside the endoplasmic reticulum and causes Ca^2 release. The nicotinic acid adenine dinucleotide phosphate (NAADP) formed from nicotinamide adenine dinucleotide (NAD) via the transglycosylation process mediated by CD38, mobilises the endolysosomes (Ly) via the activation of two-pore channels (TPC). CD38 is co-expressed as both type II and type III with its catalytic C-terminal domain facing outside and inside the cell respectively. Therefore the NAD is transported out of the cell via transporter proteins such as connexin-43 (Cnx) hemichannels and gets cyclized to form cADPR via type II CD38. The cADPR is then transported back into the cell via nucleoside transporters (NuT). Type III is responsible for the general intracellular phosphorylation processes.

2.6 Regulatory markers of T-cell activation

T-cell activation is tightly controlled by a number of mechanisms (see section 2.4.2). A disruption in the balance of inhibition and activation of T-cells could lead to a state of T-cell exhaustion. This can occur when there is a deficiency of positive co-stimulatory molecules required for successful activation or the up-regulation of negative regulators such as PD-1 and Fas (Askenasy et al., 2005; Sachdeva et al., 2010; Nakanjako et al., 2011; Shi et al., 2013, Dieterlen et al., 2014).

2.6.1 The Fas signalling pathway

Fas (also called CD95 or APO-1) is a member of the tumour necrosis factor (TNF) cell surface receptor that transduces apoptotic death signals upon activation (Felderhoff-Mueser et al., 2000; Peter et al., 2015). The Fas/FasL pathway is activated by the ligation of Fas and its ligand (FasL) (Askenasy et al., 2005). Pro-inflammatory cytokines such as TNF-α, IL-1β, IL-2, INF-$_\gamma$ and IL-24 have been reported to stimulate the Fas/FasL signalling pathway and activation-induced cell death (AICD) of T-cells (Kawakami et al., 1998; Refaeli et al., 1998; Gopalan et al., 2005). Thus, these pro-inflammatory cytokines may contribute to the chronic inflammation observed in T2DM and induce the up-regulation of Fas and Fas/FasL signalling pathway.

Fas mediated apoptosis is driven by either the intrinsic or extrinsic pathway (Fig 2.10) (Xu et al., 2016). The extrinsic apoptotic pathway is initiated by the binding of Fas to its ligand resulting in death receptor aggregation and the recruitment of adaptor proteins to the death domain (DD) (Xu et al., 2016). The subsequent activation of caspase-8 leads to the assembling of a death inducing signalling complex (DISC) (Barnhart et al., 2003), consisting of adapter protein Fas-associated death domain (FADD), Fas and cellular FLICE inhibitory protein (cFLIP) (Fulda & Debatin, 2006). The activation of the DISC results in the formation of large amounts of caspase-8 which cleaves procaspase-3 and triggers the activation of effector caspases 3, 6 and 7 (Bobé, 2002; Gordon & Kleinerman, 2010). These activated effector caspases destroy cellular and nuclear structural proteins resulting in apoptosis (Bobé, 2002).

The intrinsic pathway is activated by internal stimuli such as DNA damage (Xu et al., 2016). This pathway is initiated by mitochondrial release of cytochrome c and Smac/Diablo, which intensify caspase-3 concentration (Barnhart et al., 2003; Gordon & Kleinerman, 2010).

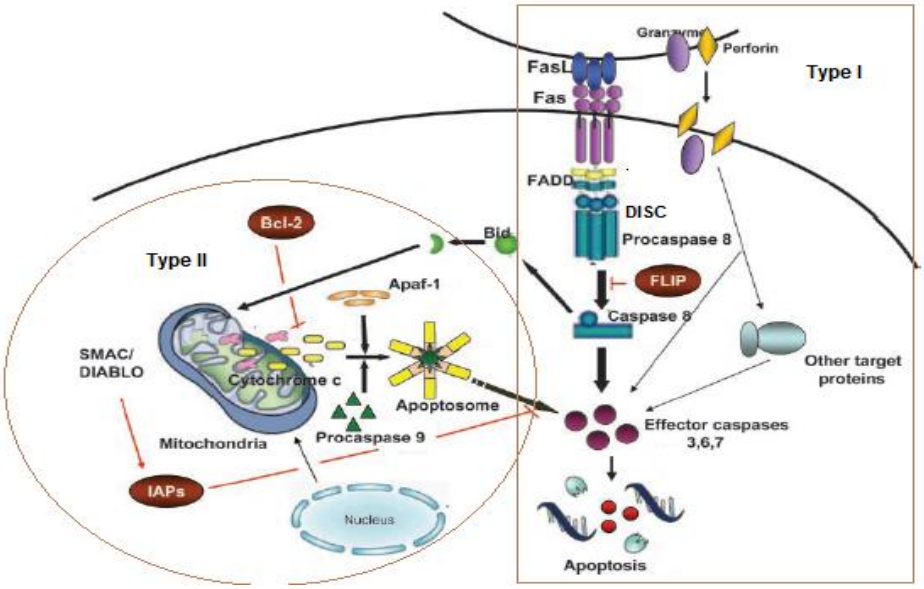

Figure 2.10: Fas apoptosis signalling pathway (adapted from Gordon & Kleinerman, 2010). The extrinsic (Type I) pathway is triggered by the ligation of Fas and its ligand FasL. This results in the formation of a death-inducing signalling complex (DISC) and subsequent activation of caspase-8. The activated caspase-8 cleaves effector caspases 3, 6 and 7 resulting in apoptosis. The intrinsic (Type II) pathway is triggered by internal stimuli and results in the formation of very little DISC and caspase-8 which is amplified by the formation of an apoptosome. The caspase-8 induces the mitochondria to release pro-apoptotic molecules cytochrome c and Smac/Diablo that intensifies caspase-3 concentration resulting in apoptosis.

Although AICD is a tightly regulated process, in chronic inflammatory conditions such T2DM, the upregulation of Fas and PD-1 expression on activated T-cells inhibits the immune response and may lead to T-cell exhaustion. (Nakanjako et al., 2011; Feuth et al., 2013) and T-cell dysfunction (Cope, 2002; Schietinger & Greenberg, 2014)

2.6.2 Programmed cell death 1 (PD-1)

Programmed cell death 1 (CD279) is an immunoreceptor belonging to the CD28/CTLA-4 family that delivers a negative signal upon interacting with its two ligands programed ligand 1 (PD-L1) or programed ligand 2 (PD-L2) (Sharpe et al., 2007; Jin et al., 2011). The PD-1 receptor is expressed on activated T and B lymphocytes, monocytes, macrophages and dendritic cells (Sharpe et al., 2007; Chinai et al., 2015). Thus, the expression of PD-1 on $CD4^+$ and $CD8^+$ T-cells is indicative of on-going cell activation (Okazaki & Honjo, 2007; Liechtenstein et al., 2012).

2.6.2.1 PD-1 signalling pathway

The PD-1 pathway is triggered by binding to its ligands (PD-L1 and PD-L2) and stimulates a range of immunoregulatory functions (Jin et al., 2011). These include tolerance, T-cell function in autoimmunity, chronic viral infections, chronic inflammation and anti-tumour immunity (Ansari et al., 2003, Freeman et al., 2006; Sharpe et al., 2007; Shi et al., 2013). The primary effect of the PD-1 pathway is not to induce apoptosis (Chinai et al., 2015) but to inhibit TCR signalling and other essential co-stimulatory signals (Fig 2.6).

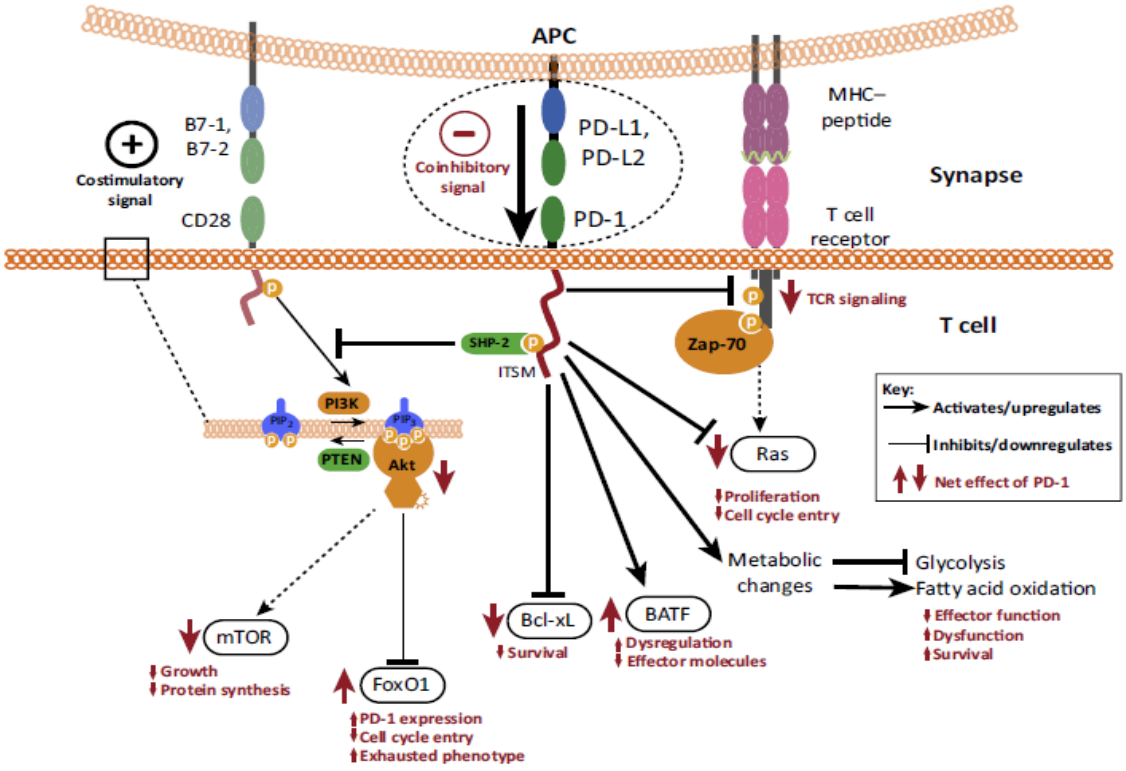

Figure 2.11: Programmed cell death 1 (PD-1) signalling pathway in T-cell function (adapted from Chinai et al., 2015). The engagement of PD-1 with its ligands (PD-L1) and (PD-L2) during antigen presentation causes the phosphorylation of immunoreceptor tyrosine-based switch motif (ITSM). The ITSM recruits SHP-2 which dephosphorylates and inactivate ZAP-70 (essential for TCR transduction signalling) and the Ras pathway resulting in the blockage of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway. The blockage of the co-stimulatory signalling PI3K pathway promotes the transcription of Forkhead box protein 01 (Fox01) factor and the downregulation of the growth regulator mechanistic target of rapamycin (mTOR) resulting in the inhibition of cell growth and cytokine secretion.

The engagement of PD-1 with its ligands leads to inhibition of TCR transduction signalling by inhibiting the phosphorylation of CD3ζ chain and Zap-70 resulting in the downregulation of the Ras pathway responsible for cell survival and proliferation (Fig 2.11) (Chinai et al., 2015; Wherry & Kurachi, 2015). In addition, PD-1 signalling initiates the phosphorylation of intracellular immunoreceptor tyrosine-based switch motif (ITSM) and the immunoreceptor tyrosine-based inhibitory motif (ITIM) (Liechtenstein et al., 2012).These phosphorylated motifs initiate the recruitment of tyrosine phosphates, SHIP-1 and/or SHIP-2 which block the co-stimulatory signalling phosphatidylinositol 3-kinase (PI3K)/Akt pathway (Xing & Hogquist, 2012; Chinai et al., 2015; Wherry & Kurachi, 2015). This pathway is crucial in delivering the CD28 co-stimulatory signal essential for T-cell activation (Jin et al., 2011).

The blockage of the PI3K pathway increases the half-life of the transcription factor Forkhead box protein 01 (Fox01) and downregulates the growth regulator mechanistic target of rapamycin (mTOR) which collectively leads to the upregulation of PD-1 expression (Chinai et al., 2015). Therefore, the PD-1 pathway provides co-inhibitory signalling that blocks CD28-mediated activation of the PIK3 pathway resulting in reduced Akt phosphorylation and glucose metabolism in T-cells (Freeman et al., 2006).

2.6.2.2 Regulation of PD-1 expression

The expression of PD-1 is upregulated on T-cells by TCR-mediated signalling and is amplified by tumour necrosis factor (TNF) (Jin et al., 2011). Cytokine signalling plays a very critical role in regulating the expression of PD-1. The release of IL-2, IL-7, IL-15 and IL-21 up-regulates PD-1 expression on T-cells (Chinai et al., 2015). Furthermore, IL-6 and IL-12 increases PD-1 expression by promoting the transcription of *Pdcd1* gene that codes for PD-1 (Austin et al., 2014). Transcription factors such as T-bet directly repress transcription of *Pdcd1* gene (Kao et al., 2012).

However, in chronic inflammation, T-bet is downregulated promoting increased interaction of FOX01 and the PD-1 promoter region resulting in the up-regulation of PD-1 expression (Chinai et al., 2015). When T-cells are activated, members of the Nuclear Factor of Activated T-Cells (NFAT) such as NFATc1 translocate into the nucleus and initiates the transcription of the *Pdcd1* gene leading to the up-regulation of PD-1 expression (Oestreich et al., 2009; Austin et al., 2014). Therefore, the expression of PD-1 on T-cells is tightly regulated by a combination of transcription factors and cytokine signalling (Fig 2.12).

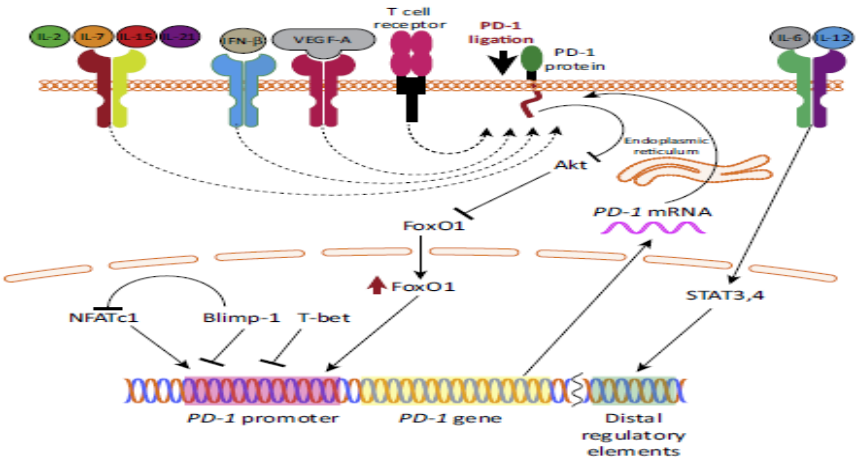

Figure 2.12: Regulation of PD-1 expression by transcriptional factors and cytokine signalling (adapted from Chinai et al., 2015). Cytokine signalling via interleukin (IL) 2, IL-7, IL-15 and IL-21 upregulates the expression of PD-1. Furthermore, IL-6 and IL-12 acting via signal transducer and activator of transcription (STAT) 3 and 4 respectively, up-regulated PD-1 expression by activating the *Pdcd1* gene via distal regulatory elements. Transcriptional factors such as Nuclear Factor of Activated T-Cells (NFAT)-c1 and Forkhead box protein 01 (Fox01) promotes the up-regulation of PD-1 by promoting the transcription of *Pdcd1* gene. T-bet and Blimp-1 provides the negative inhibition of PD-1 by blocking the transcription of the *Pdcd1* gene.

2.7 T-cell function in inflammatory conditions.

Antigen activation of naïve T-cells is key in the activation of adaptive immunity (Bonilla & Oettgen, 2010). A fine balance between stimulatory and inhibitory regulation signals is essential in maintaining immune homeostasis (Felderhoff-Mueser et al., 2000). However, in T2DM, due to chronic inflammation, which is characterised by elevated levels of cytokine and chemokines, the cell mediated signalling is persistently dysregulated (Lontchi-Yimagou et al., 2013; Ozougwu, 2013). Thus, this dysregulation leads to chronic immune activation characterised by upregulation of immune activation markers such as CD38 and HLA-DR on T-cells (Smith et al., 2013).

After prolonged activation, the balance between activation antigens and inhibitory receptors (FAS/PD-1 and others) is disrupted and ultimately leads to AICD (Freeman et al., 2006; Sachdeva et al., 2010; Ward, 2010). This may also result in T-cell exhaustion, which is characterised by the down regulation of positive co-stimulatory molecules such as CD28 and the up-regulation of negative co-stimulatory molecules such as CTLA-4, PD-1 and Fas (Askenasy et al., 2005; Sachdeva et al., 2010; Nakanjako et al., 2011; Shi et al., 2013, Dieterlen et al., 2014).

2.7.1 CD38 and HLA-DR in inflammatory conditions

In inflammatory bowel disease (IBD), elevated levels of HLA-DR and CD38 on both $CD4^+$ and $CD8^+$ T-cell have been reported (Funderburg et al., 2013). Furthermore, these were associated with increased levels of pro-inflammatory cytokines (IL-2, IL-6, interferon $_\gamma$) and C-

reactive protein (CRP), which led to the conclusion that the damage to the gastrointestinal tract in this condition may be due to dysregulated chronic T-cell activation (Funderburg et al., 2013). Elevated expression of these markers on the surface of $CD4^+$ and $CD8^+$ T-cells have also been described in individuals with Systemic lupus erythematosus (SLE), a chronic autoimmune disorder which is also characterised by inflammation and cytokine release imbalances (Viallard et al., 2001; Pavon et al., 2013). Increased HLA-DR expression on circulating T-cells has also been reported in dilated cardiomyopathy, implicating chronic inflammation as a contributing factor to the ongoing immune response observed in this condition (Ueno et al., 2007).

The implications of inflammation and immune activation in human immunodeficiency virus (HIV) infection has been extensively reported in the literature (Nakanjako et al., 2011; B. Nkambule et al., 2015). The HIV induces a chronic inflammatory state that triggers immune activation characterised by increased levels of pro-inflammatory cytokines and chemokines and an up-regulation in the expression of immune activation markers (such as CD38 and HLA-DR) on T-cells (Smith et al., 2013). Despite the use of antiretroviral (ARV) drugs, HIV seropositive individuals still display increased T-cell activation (B. Nkambule et al., 2015), albeit lower than in ARV naïve HIV seropositives (Hunt et al., 2008). The abnormally highly activated ($CD38^+$ $HLA\text{-}DR^+$) T-cells observed in HIV infection is assumed to contribute to progressive CD4 T-cell loss and progression to Acquired Immune Deficiency Syndrome (AIDS) related death (Hunt et al., 2008; Smith et al., 2013).

Increased levels of HLA-DR and CD38 expression on both $CD4^+$ and $CD8^+$ T-cells have also been reported in HIV/Hepatitis C virus (HCV) co-infection indicating increased immune activation and poor prognosis (Feuth et al., 2013). In addition, high levels of alanine transaminase (ALT), produced by the liver due to HCV infection have been correlated with the up-regulated expression of CD38 and HLA-DR on T-cells (Feuth et al., 2013).

2.7.2 PD-1 and Fas in inflammatory conditions

Viruses exploit the PD-1/PD-L pathway to evade host immune effector mechanisms by inducing anergy in virus-specific $CD8^+$ T-cells (Fig 2.13) (Butte et al., 2007; Sharpe et al., 2007). This has been described in chronic HIV infection, in which specific effector $CD8^+$ T cells showed a reduced capacity to produce cytokines and proliferate (Trautmann et al., 2006). High immune exhaustion measured by increased levels of PD-1 on $CD4^+$ and $CD8^+$ T-cells has been reported in HIV seropositive individuals (Trautmann et al., 2006; Nakanjako et al., 2011). In hepatitis B virus (HBV) infection, upregulated levels of PD-1 on both $CD4^+$ and $CD8^+$ intrahepatic mice T-cells has also been described (Tzeng et al., 2012). These studies indicate that PD-1 expression on T-cells may serve as a useful and robust marker to measure T-cell exhaustion in chronic immune activation states.

The expression of CD95 (Fas) on T-cells has been described in several studies as a pro-apoptotic receptor in AICD (Bäumler et al., 1996; Tanner & Alfieri, 1999; Felderhoff-Mueser et al., 2000). In that context, Fas mediated T-cell exhaustion was reported in HIV/HCV co-infection (Feuth et al., 2013). Furthermore, increased $CD4^+$ and $CD8^+$ Fas mediated apoptosis has also been described in HIV infection (Bäumler et al., 1996; Merlini et al., 2012). The depletion of $CD4^+$ in HIV infections is believed to contribute significantly to the immunodeficiency observed in AIDS (Peter et al., 1997). Fas mediated T-cell apoptosis has further been described in Epstein-Barr viral infection (Tanner & Alfieri, 1999).

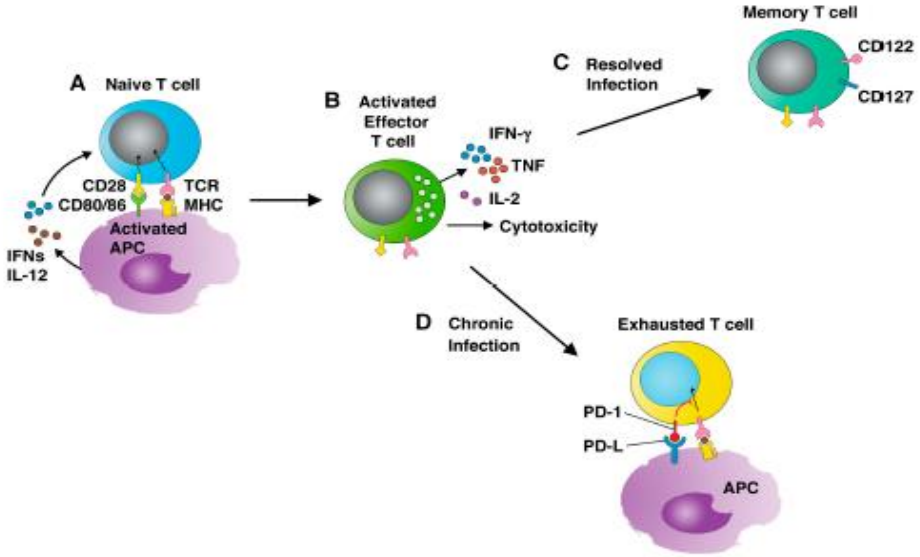

Figure 2.13: PD-1-PD-L pathway inducing T-cell dysfunction in chronic viral inflammation (adapted from Freeman et al., 2006). **A)** Activated antigen presenting cell (APC) express the CD80/CD86 receptor complex that binds to CD28 expressed on the surface of naïve T-cells. This leads to the activation of T-cells and differentiation into effector T-cells. **B)** Upon activation, T-cells proliferate and provide effector responses by enhanced cytotoxic activity and pro-inflammatory cytokine production. **C)** Once the infection or stimulus resolves, long-lived memory T-cells are formed which express cell surface CD122 and CD127. **D)** However, if infection persists, as in the case of a chronic viral infection, T-cells upregulate the surface expression of PD-1 which upon binding to its ligand expressed on the surface of activated APC induces T-cell exhaustion.

Therefore, it appears that in chronic inflammatory conditions, in which there is prolonged activation of T-cells, there is up-regulation and elevated expression of negative regulators such as Fas and PD-1 on T-cells leading to exhaustion. Measurement of these markers could therefore be utilised to analyse T-cell exhaustion.

2.8 Immune dysregulation and dysfunction in T2DM

Pro-inflammatory signalling from cytokines such as TNF-α, IL-1β and INF-γ have been reported to inhibit insulin signalling and induce obesity associated IR and subsequent hyperglycaemia in T2DM (Hotamisligil et al., 1995; Chng et al., 2015). Hyperglycaemia triggers pathways such as the aldose reduction pathway, advanced glycation end product (AGE) pathway, reactive oxygen intermediate pathway and PKC pathway which collectively

give rise to the production of reactive oxygen species (ROS) and inflammatory mediators (King, 2008).

The interaction between AGEs and its receptor RAGE, attracts immune cells into diffused glycated tissue and subsequently activates them to induce a pro-inflammatory response by secretion of pro-inflammatory cytokines in T2DM (Hu et al., 2015). Furthermore, the AGE-RAGE interaction upregulates RAGE expression which activates the NF-kB, PI3K/Akt and MAP kinase pathways resulting in further amplification of pro-inflammatory signals (King, 2008; Hu et al., 2015).

Altered lipolysis and necrosis of the hypertrophic adipose tissue causes an elevation of pro-inflammatory saturated fatty acids which act as ligands for Toll-like receptors (TLRs) initiating adipose tissue inflammation (Nikolajczyk et al., 2011). Interestingly, unlike in classical inflammation, the adipose tissue infiltration starts with neutrophils followed by B-cells, T-cells and finally monocytes/macrophages (Nikolajczyk et al., 2011). Hyperglycaemia has been reported to induce neutrophil dysfunction by interfering with the myeloperoxidase (MPO) (Xiu et al., 2014). Furthermore, activated neutrophils in T2DM have been reported to have elevated levels of RAGE expression, increased adherence to adipocytes and impaired phagocytic function (Nikolajczyk et al., 2011; Hu et al., 2015).

Peripheral blood monocytes in hyperglycaemia have been reported to secrete high levels of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 (Nikolajczyk et al., 2011; Xiu et al., 2014). Interleukin 6 (IL-6) has also been reported to induce insulin resistance in hyperglycaemia (Xiu et al., 2014). The activation of monocytes in T2DM has been attributed to the presence of a TLR ligand rich milieu. However, contrary to these findings, hyperglycaemia has been reported to supress HLA-DR expression on monocytes, thus impairing its antigen presenting activity (Turina et al., 2006). In support of this, another study reported that hyperglycaemia inhibits ROS production and the secretion of pro-inflammatory cytokines (IL-6, IL-8, macrophage inflammatory protein-1 and TNF-α) by monocytes (Qadan et al., 2010). These inconsistent results on the effect of hyperglycaemia on monocytes cytokine secretion may be due to different model systems used in these studies.

Macrophages are important regulators of inflammation with the “M1” macrophages classified as pro-inflammatory whilst the “M2” subset as anti-inflammatory (Nikolajczyk et al., 2011). Reports suggest that tissue macrophages in obese and T2DM individuals are activated and are major sources of pro-inflammatory cytokines (Nikolajczyk et al., 2011). In addition, both “M1” and “M2” macrophages utilise glucose and fatty acids as energy sources (Nikolajczyk et al., 2011). A further study reported on the effects of hyperglycaemia on cytokine secretion by macrophages revealed that hyperglycaemia induced the secretion of IL-1β, TNF-α (pro-

inflammatory) and IL-1Ra (anti- inflammatory) cytokines whilst suppressing the production of macrophage inflammatory protein-4 (MIP-4) (Moganti et al., 2016). These studies suggest that hyperglycaemia could induce an unbalanced production of inflammatory cytokines which may contribute to the chronic inflammatory state observed in individuals with hyperglycaemia.

Therefore, low grade chronic inflammation, cytokine generation and the activation of innate immune cells play an important role in the development of insulin resistance and the pathogenesis of T2DM (Pickup & Crook, 1998; King, 2008; Kumar et al., 2014; Chng et al., 2015; Moganti et al., 2016). However, recent findings have revealed that these processes are not only limited to the innate immune response but also involves the adaptive immune response mediated by T-cells (Wu et al., 2011; Fang et al., 2015)

2.8.1 T-cell function in hyperglycaemia

Hyperglycaemia in T2DM has been reported to induce macrophages to produce IL-12 which stimulates the $CD4^+$ T-cell subset to produce INF-ɣ which in turn primes macrophages to stimulate the T-helper (Th) pro-inflammatory subsets (Th1 and Th17) (Wu et al., 2011; Ip et al., 2015). The frequency of these circulating Th pro-inflammatory subsets is increased compared to T_{regs}, thus resulting in a systemic pro-inflammatory state in T2DM (Wu et al., 2011; Chng et al., 2015). A flow cytometry based study on the effects of hyperglycaemia on myocardial infarction (MI) showed that newly diagnosed and hyperglycaemic patients with MI who had not received therapy, had higher expression of HLA-DR on T-cells (both $CD4^+$ and $CD8^+$ subsets) and increased CRP levels compared to the normoglycaemic patients (Marfella et al., 2003). These results suggest that hyperglycaemia may cause activation of T-cells. However, the diagnosis of hyperglycaemia in this study was done solely based on random glucose levels upon admission to the emergency department. This criterion did not take into account factors such as diet which may elevate the glucose levels.

In support of these findings, elevated levels of T-cell pro-inflammatory cytokines (IL-2, IL-1β and TNF-α) was reported in hyperglycaemia-induced T-cells from healthy non-diabetic individuals (Stentz & Kitabchi, 2005). Although these findings suggests that hyperglycaemia may cause further activation of the immune system by pro-inflammatory cytokine release, the sample size of only five (5) participants was too small to make conclusions. Interestingly, this study also revealed that hyperglycaemia induces the development of insulin signalling protein such as insulin receptor substrate 1 (IRS-1) on T-cells which may be a protective mechanism against glucotoxicity (Stentz & Kitabchi, 2005). Further studies are required to investigate T-cell insulin receptors in chronic hyperglycaemia.

The secretion of the pro-inflammatory cytokine IL-17 by $CD4^+$ T-cells in T2DM has been reported to trigger the NF-kB pathway and stimulate granulocytes (Nikolajczyk et al., 2011). Collectively, these studies suggest that hyperglycaemia may induce the activation of T-cells with the release of pro-inflammatory cytokines which ultimately results in chronic inflammation.

In contrast to the above, decreased tyrosine-phosphorylation was reported in hyperglycaemic Jurkat T-cells compared to an untreated control group (Boldizsar et al., 2002). This flow cytometry based study reported glucose concentration-depended elevation of basal cytosolic free calcium levels at a baseline state. Both these findings resulted in abnormal calcium homeostasis and reduction in calcium mediated T-cell transduction signalling which is essential for the activation of T-cells (Guy & Vignali, 2009). This study therefore suggested that hyperglycaemia inhibits T-cell activation by disrupting calcium transduction signalling. However it is essential to note that the T-cells used in this study were Jurkat cells and may not reflect normal T-cells *in vivo*. Based on these reports, the activation state of T-cells in T2DM still requires investigation. The use of T-cell activation markers such as HLA-DR and CD38 may help clarify if the T-cell dysfunction in T2DM is due to hyperglycaemia inducing or inhibiting T-cell activation.

The dysregulation of T-cell negative co-stimulatory factors have been implicated in the pathogenesis of T2DM. Upregulation of PD-1 on $CD4^+$ T-cells and their subsequent resistance to apoptotic signalling was described as a contributing factor to T-cell dysfunction in T2DM (Shi et al., 2013). In a nonobese diabetic (NOD) mouse model, inhibition of the PD-1-L pathway by addition of anti PD-1 and anti-PD-L1 to pre-diabetic NOD mice, led to rapid and exacerbated diabetes with both $CD4^+$and $CD8^+$ T-cells releasing increased concentrations of pro-inflammatory factors (INF-$_\gamma$ and TNF), responsible for insulitis in T1DM (Ansari et al., 2003). Thus, the PD-1 pathway is crucial in regulating effector T-cell function by inhibiting auto-immune responses in T1DM.

A study investigating the expression of PD-1 on T-cells in known T2DM and severe sepsis reported an upregulation of PD-1 on $CD4^+$ and $CD8^+$ circulating T-cells in both conditions compared to healthy controls (Jia et al., 2016). These findings suggest that there is a significant level of immune exhaustion in T2DM and severe sepsis, which predispose individuals to infections. In concordance with these findings, increased levels of PD-1 expression on lymphocytes was reported in both proliferative diabetic retinopathy and known T2DM cases compared to normoglycaemic controls (Fang et al., 2015). Interestingly, the same study reported decreased messenger RNA (mRNA) expression of PD-1 in both conditions compared to the normoglycaemic group using real time polymerase chain reaction (PCR) (Fang et al., 2015). It is important to note that this study analysed PD-1 expression on

all lymphocytes and did not isolate any specific T-cell subset. Collectively, these findings support the theory that hyperglycaemia is involved in the initiation of diabetic pathologies and may induce activation-induced apoptosis of lymphocytes by PD-1.

Contrary to this, a further study showed that hyperglycaemia inhibits the expression of CTLA-4 (a negative co-stimulatory marker) on T-cells (Marfella et al., 2003). This further supports the findings that hyperglycaemia interferes with the autoregulation of activated T-cells. However, this raises a question; “Does hyperglycaemia cause an increase or decrease in the expression of negative co-stimulatory markers on T-cells”?

These contradictory findings in the literature could be explained by differences in the population groups, medication and methodologies. Therefore, further research is required to investigate the effects of hyperglycaemia on the activation and control of T-cell function.

Fas-induced lymphocytes and pancreatic β-cell death has been well described in the pathogenesis of T1DM (DeFranco et al., 2001; Cnop et al., 2014). In T2DM, an upregulation of Fas expression in the adipose tissue and pancreatic β-cells has been reported to cause insulin insensitivity, hyperglycaemia and pro-inflammatory cytokine release (Nolsøe et al., 2006; Blüher et al., 2014). Although these studies indicate Fas involvement in the inflammation process and immune dysfunction in T2DM, studies investigating the effect of hyperglycaemia on Fas expression on T-cells in T2DM are limited. Therefore this study aims to investigate this unknown phenomenon.

2.9 Conclusions

The effect of hyperglycaemia on cells of the innate immune system in T2DM has been well described and implicated in the progression of the disease and the development of complications such as increased susceptibility to infection and thrombotic events. However, studies investigating the adaptive immune response, mediated by T-cells, and their role in the pathogenesis still remain scarce and controversial.

The T-cell mediated adaptive immune response has been well described in the pathogenesis and progression of various other chronic inflammatory conditions. Notably, the continuous activation of T-cells marked by expression of activation markers and their subsequent release of pro-inflammatory cytokines has the potential to induce T-cell exhaustion and initiate AICD. Therefore, the investigation of T-cells in hyperglycaemic conditions could provide further insight into the immune dysfunction in T2DM as well as assist in identifying pathways which could be targeted in disease management and treatment.

The aim of this study is to investigate markers of chronic immune activation and T-cell function in hyperglycaemia and to test the hypothesis; “Hyperglycaemia in uncontrolled

hyperglycaemic individuals induces a low grade chronic inflammatory state that causes continuous activation of T-cells which may result in immune dysregulation and exhaustion".

CHAPTER THREE
MATERIALS AND METHODS

3.1 Participants and Sampling

Sixty nine (69) participants of mixed ancestry were recruited from the Bellville South, Ward 9 area, under the Tygerberg District of Bellville in the Western Cape, South Africa. Informed consent was obtained from participants (see Addendum 1) and this project forms part of an ethically approved (CPUT/HW-REC 2013/H44) larger study. Ethical clearance was obtained for this study from the Cape Peninsula University of Technology (CPUT) research ethics committee; Ref No CPUT/HW-REC 2015/H26 (see Addendum 2). The study was conducted according to the Code of Ethics of the World Medical Association (Declaration of Helsinki 2013) and all participants signed written informed consent after all the procedures had been fully explained in the language of their choice. Computer generated codes were used to identify all participants in order to protect their confidentiality and to avoid bias. The glucose status of all participants was not revealed to the researcher until after data analysis.

3.1.1 Inclusion Criteria

Participants included in this study gave informed consent, were over 20 years of age, fasting, not pregnant and had not taken any medication for recent infection.

3.1.2 Exclusion Criteria

Participants were excluded if they showed and/or reported any evidence of; current or recent acute illness, co-existing chronic illness, use of steatogenic medications or drugs that interfere with the immune system and family history of atopy. This was achieved by use of questionnaires and interviews.

3.1.3 Sample Collection

Fasting and two hours post the oral glucose tolerance test (OGTT), venous blood was collected into 4.5ml and 10ml tubes of EDTA, sodium citrate and serum separator tube (SST) by qualified nurses/phlebotomists. Blood collected in sodium citrate was used in flow cytometry analysis as it has been reported to be suitable for T-cell activation studies (Hoffmeister et al., 2003; Mallone et al., 2011). Blood drawn in EDTA, SST and sodium citrate (10ml) was used for biochemical and routine haematological analysis. Samples were kept at room temperature to avoid any effects of temperature fluctuations on T-cell viability and activation. The time frame from sample collection to analysis was limited to 1-2 hours.

3.1.4 Clinical Measurements

Clinical parameters were measured by trained clinical personnel and included weight, height, hip-to-waist circumference and blood pressure. Other parameters such as the body mass index (BMI) were calculated using the formula BMI = $\frac{Body\ mass\ (Kg)}{height\ (m)^2}$ (Catalán et al., 2007).

3.1.5 Biochemical Measurements

Biochemical and routine haematological parameters tests were performed at the National Heath Laboratory Service (NHLS) and Pathcare laboratories which are both accredited by the South African National Accreditation Systems (SANAS). The analytes and parameters measured are shown in table 3.1. Other parameters measured were serum CRP levels, full lipid profile, white cell differential count and full blood counts (FBC).

Table 3.1: Biochemical and routine haematological parameters measured

Analyte	**Method**	**Equipment used**
Plasma glucose (mmol/L)	Enzymatic Hexokinase	Beckman AU (Beckman Coulter, Miami, USA)
HbA1c (%)	High Performance Liquid Chromatography	Bio-Rad Variant Turbo (Bio-Rad, California, USA)
Insulin (mmol/L)	Paramagnetic Particle Chemiluminescence Assay	Beckman DXI (Beckman Coulter, Miami, USA)
Lymphocyte Absolute count	Impedance Method	Coulter LH 750 Haematology Analyser (Beckman Coulter, Miami, USA)
Lipids Profile (mmol/L)	Friedwald's Formula	Cobas 600 Roche Diagnostics (Indiana, USA)
Full blood count (cells/µl)	Impedance Method	Coulter LH 750 Haematology Analyser (Beckman Coulter, Miami, USA)
Ultra-sensitive (U-CRP)	Latex Particle Immunoturbidimetric	Beckman AU (Beckman Coulter, Miami, USA)

3.2 Flow Cytometry Analysis

3.2.1 Instrument Setup

Data acquisition was performed using a Beckman Coulter 8-colour Navios flow cytometer (Beckman Coulter, Miami, FL). Flow check pro fluorescent labelled beads of known size from Beckman Coulter were used for daily quality control (QC) in order to verify the optical path and laminar flow of the cytometer (See Appendix 1). This was done to ensure that the reporting of standardised results was not influenced by incidental changes or long-term instrumental drifts.

3.2.2 Detector Settings

Forward scatter (FS) and side scatter (SS) parameters measured by silicon diodes and photomultiplier tubes (PMTs) respectively, were set at a linear-scale. This enabled the separation of lymphocytes, monocytes and granulocytes based on cell size and internal complexity or granularity (Fig 3.1A). An unstained fresh blood sample was used to set the quadrants for all flow channels. Compensation beads (VersaComp Antibody Capture bead Kit from Beckman Coulter) were used to determine the percentage of spectral overlap and to perform colour compensation. Stained $CD3^+$ lymphocytes were used as the primary gate for T-cells (Fig 3.1B).

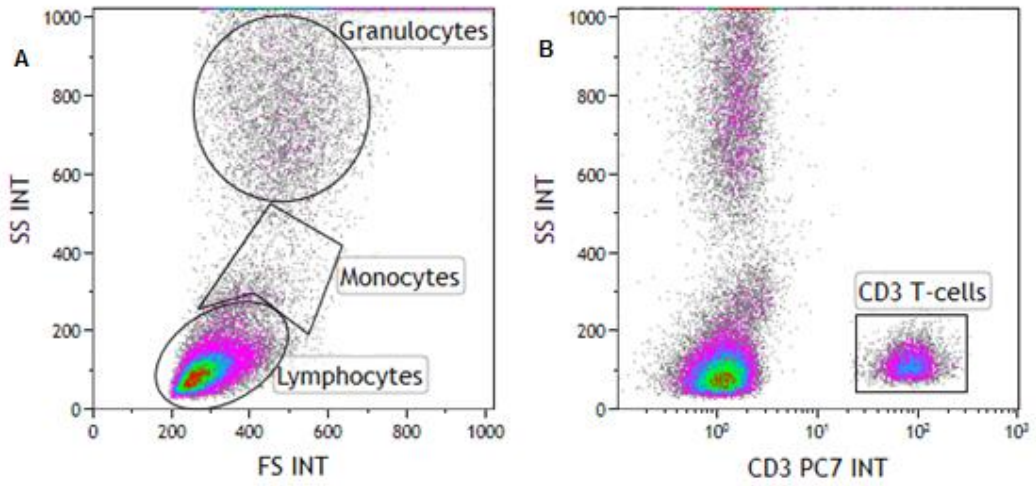

Figure 3.1: (A) Forward scatter (FS) and side scatter (SS) parameters of unstained sample at linear-scale. **(B)** CD3-PC7 was used as a primary T-cell marker to discriminate T-lymphocytes from other haemopoietic cells.

3.2.3 Antibodies and Reagents

The anti-human CD3-PC7 (clone IM2469), CD4-PE (clone 13B8.2), CD8-APC (clone B9.11), CD95-FITC (clone UB2) antibodies were obtained from Beckman Coulter, Miami, FL. Anti-human HLA-DR-Alexa Fluor 700 (clone L243), PD-1-APC (clone EH 12.2H7) antibodies and Zombie NIR dye- APC/Cy7 were obtained from BioLegends, San Diego, CA. CD38-PerCP-

Cy5.5 (clone HIT2) was obtained from Becton, Dickinson, New Jersey . Phosphate buffered saline (PBS) without Ca^{2+} or Mg^{2+} was obtained from Lonza, Belgium and VersaLyse buffer from Beckman Coulter, Miami, FL.

3.2.4 Fluorescence Minus One (FMO)

Fluorescence minus one (FMO) controls were used to accurately identify positive events by discriminating cells that exhibited fluorescence signals from those that did not (see Appendix 2). An FMO control sample contains all other flourochrome-conjugated antibodies except for the one of interest. Positive events were defined by a higher mean fluorescence intensity (MFI) value than that of the negative MFI. The use of an FMO control was important in this study as it enabled the identification of cell surface markers that can be up-regulated or down-regulated as well as the discrimination of low signals when the positive population does not separate well from the negative. Furthermore, quadrants were then set close to the negative population as possible to exclude non-specific binding and/or auto-fluorescence.

3.3. Optimisation

3.3.1 Antibody Titration

Five (5) different concentrations of each monoclonal antibody (mAb) were used to determine the optimal concentration of each antibody to be used when making up the mAb cocktail. Signal to noise ratios were then calculated using formula; Signal to noise ratio = $\frac{Positive\ MFI}{Negative\ MFI}$ x *100* (Hulspas, 2010). The volume with the highest signal to noise ratio was chosen as the optimum mAb volume titration for each particular antibody used in a specific panel (see Appendix 3).

3.3.2 Glucose Incubation Time

Fifty microliters (50µl) of sodium citrate blood from three samples was added to 20µl of three different glucose concentrations (2mmol/l, 5mmol/l and 30mmol/l) mimicking hypoglycaemia, normoglycaemia and hyperglycaemia respectively (Oleszczak et al., 2012). Four 15 minutes incubation time intervals (15, 30, 45 and 60 minutes) were investigated to determine the best optimum glucose incubation time for the functional test protocol prior to the staining procedure. This was achieved by measuring percentages of T-cell (CD4) recovery (see Appendix 4). An incubation period of 45 minutes was selected and used as the optimum glucose incubation time.

3.3.3 T-Cell Viability Control Test

Zombie NIR-APC/Cy7 fixable viability kit dye was used to verify the viability of cells and T-cell recovery post the 45 minutes incubation. The T-cell viability control test demonstrated

that the incubation time and staining techniques applied in this study did not influence cell survival (see Appendix 5).

3.4. Immune Measurements

3.4.1 Baseline Protocol

3.4.1.1 Baseline measurements of T-cell antigen expression

In order to determine the baseline levels of immune activation, 50µl of citrated whole blood was pipetted into two tubes. The first tube was stained with 5µl of a mAb cocktail containing CD3-PC7 (clone IM2469), CD4-PE (clone 13B8.2), CD8-APC (clone B9.11), CD38-PerCP-Cy5.5 (clone HIT2), HLA-DR-Alexa Fluor 700 (clone L243) and CD95-FITC (clone UB2). The second tube was stained with CD3-PC7 (clone IM2469), CD4-PE (clone 13B8.2), CD38-PerCP-Cy5.5 (clone HIT2), CD95-FITC (clone UB2) and PD-1-APC (clone EH 12.2H7). Thereafter, the mixture was incubated at room temperature in the dark for 15 minutes. Five hundred microliters (500µl) of VersaLyse lysing buffer (Beckman Coulter, Miami, FL) was then added (to lyse RBCs) and incubated at room temperature in the dark for a further 15 minutes. Thereafter, 500µl of dilution buffer (PBS without Ca^{2+} or Mg^{2+}) (Lonza, Belgium) was added and the samples were acquired using the 8-colour Navios flow cytometer (Beckman Coulter, Miami, FL). The Kaluza analysis software (V1.3) (Beckman Coulter, Miami, FL) was used for data analysis. The percentage expression and MFI of the selected markers were then recorded.

3.4.2 Incubation protocol

3.4.2.1 The effects of glucose on T-cell antigen expression

A total of 31 participants (16 with hyperglycaemia and 15 controls) from the total population of 69 were randomly selected into this protocol. In order to evaluate the effects of glucose on T-cell antigen expression, 20µl of three different glucose concentrations (2mmol/l, 5mmol/l and 30mmol/l) mimicking hypoglycaemia, normoglycaemia and hyperglycaemia respectively (Oleszczak et al., 2012) were used. Briefly, 50µl of citrated whole blood was incubated at 37^{o}C for 45 minutes in the presence of glucose at varying concentrations (2mmol/l, 5mmol/l and 30mmol/l). The samples were then stained using 5µl of mAb cocktail containing CD3-PC7 (clone IM2469), CD4-PE (clone 13B8.2), CD8-APC (clone B9.11), CD38-PerCP-Cy5.5 (clone HIT2), HLA-DR-Alexa Fluor 700 (clone L243), CD95-FITC (clone UB2) and PD-1-APC (clone EH 12.2H7). The mixture was then incubated at room temperature in the dark for 15 minutes. Five hundred microliters (500µl) of VersaLyse lysing buffer (Beckman Coulter, Miami, FL) was then added and incubated at room temperature in the dark for 15 minutes. Thereafter, 500µl of PBS (without Ca^{2+} or Mg^{2+}) (Lonza, Belgium) was added and the samples were then loaded into the 8- colour Navios flow cytometer (Beckman Coulter) for

acquisition. The Kaluza analysis software (V1.3) (Beckman Coulter, Miami, FL) was used for data analysis.

3.4.3 Gating Strategy

An unstained fresh blood sample was used as the negative control in all histograms to correct for autofluorescence as well as distinguishing positive from negative events (Fig 3.2C-F). The T-cell population was identified by using CD3-PC7 and SS (Fig 3.2A) and T-helper and T-cytotoxic cells were identified as $CD3^+CD4^+$ and $CD3^+CD8^+$ respectively (Fig 3.2B). The percentage (%) expression and MFI of CD38, HLA-DR, CD95 and PD-1 on T-cells were assessed and recorded. A total of 10 000 events were acquired per sample and in the case of those with low cell count, acquisition was done up to 3 minutes.

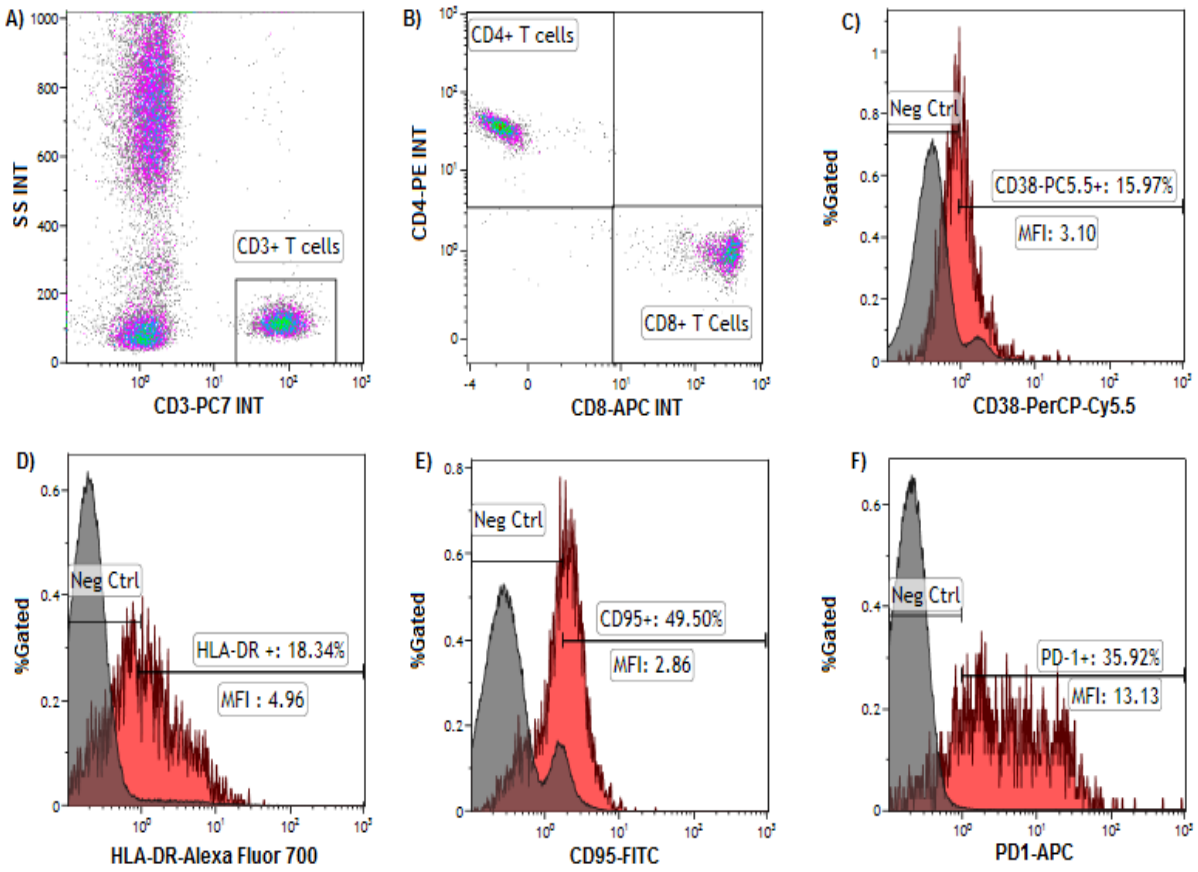

Figure 3.2: T-cell gating strategy. Plot **A** Illustrates the primary gate for T lymphocytes using the side scatter properties and $CD3^+$ events. Plot **B** shows further sub-classification of T-cells into T-helper cells ($CD3^+CD4^+$) and cytotoxic T-cells ($CD3^+CD8^+$). **C-F)** The histograms illustrates how positive events were determined from negative events using FMOs. The percentage expression and MFI of HLA-DR **(C)**, CD38 **(D)**, PD-1 **(E)** and CD95 **(F)** on T-cells were then recorded.

3.6 Statistical Analysis

Statistica statistical software was used for all statistical analysis. Medians and/or means were used as measures of central location for ordinal and continuous responses and standard deviations and quartiles as indicators of spread. Relationships between two continuous

variables were analysed with regression analysis and the strength of the relationship measured with the Pearson correlation and where the continuous variables were not normally distributed, Spearman correlation was used. When one continuous response variable was related to several other continuous input variables, multiple regression analysis was used and the strength of the relationship was measured with multiple correlations.

For randomized non-parametric designs, the Mann-Whitney U test or the Kruskal-Wallis test was used and data was presented as median and interquartile ranges. The student t-test was used for parametric data and results presented as mean (±2SD) and standard deviation. The relationship between two nominal variables was investigated with contingency tables and likelihood ratio chi-square tests. A p-value of $p < 0.05$ represented statistical significance in hypothesis testing and 95% confidence intervals were used to describe the estimation of unknown parameters.

CHAPTER FOUR

RESULTS

4.1 Individual demographics

This cross-sectional study included a total of 69 participants from Bellville South, Western Cape, South Africa. Of these, 34 were hyperglycaemic, that is with a 2hr oral glucose tolerance test (OGTT) value of $\geq$ 7.8mmol/l (140mg/dl) and included individuals with DM, IFG and/or IGT according to the WHO classification and 35 were normoglycaemic (WHO, 2006). The median age of the normoglycaemic group was 53.0 years (40.0-61.5) and 56.0 years (52.0-62.0) in the hyperglycaemic group, p=0.0567. The median BMI was above the normal reference range (18.50-24.99) in both groups with a value of 29.2 (24.4-34.5) in normoglycaemia compared to 31.3 (29.3-34.9) in the hyperglycaemic group, p=0.1041 (see table 4.1). A total of 14% of the participants were male and 86% were female.

Table 4.1: Participants demographics

Parameter	**Total N69**	**Normal N35**	**Hyperglycaemia N34**	**P=value**
	Median (25Q-75Q)			
Age (years)	56.0 (47.0-62.0)	53.0 (40.0-61.5)	56.0 (52.0-62.0)	0.0567
BMI	30.5 (26.2-34.8)	29.2 (24.4-34.5)	31.3 (29.3-34.9)	0.1041
Waist (cm)	97.5 (88.5-105.0)	94.0 (80.3-102.8)	100.9 (92.8-106.8)	0.0866

4.2 Biochemical parameters

The hyperglycaemic group had significantly elevated fasting glucose levels with median values of 5.90mmol/L (5.40-6.20) compared to the normoglycaemic group with a value of 5.10mmol/L (4.65-5.40), p<0001. In addition, the 2 hour oral glucose tolerance test (OGTT) was significantly increased in the hyperglycaemic group 9.25mmol/L (8.40-11.00) compared to the normoglycaemic group 5.85mmol/L (4.85-7.00), p<0.0001. The HbA1c levels were also significantly increased in the hyperglycaemic group at 6.30% (6.00-6.60) compared to the normoglycaemic group 5.75% (5.40-6.05), p < 0.001.

Likewise, the median insulin levels were significantly higher in the hyperglycaemic group 9.95mIU/L (7.20-16.30) compared to the normoglycaemic group 6.55mIU/L (4.10-9.35), p=0.0092. Median values for ultra-sensitive C-reactive protein (CRP) and lipids (triglycerides, LDL Cholesterol and HDL cholesterol) were higher in the hyperglycaemic group than in the normoglycaemic group. However, there was no statistical significant difference between the two groups of individuals (see Table 4.2).

Table 4.2: **Biochemical parameters**

Parameter	Reference ranges	Median (25Q-75Q)		P=value
		Normal N35	Hyperglycaemia N34	
Fasting Glucose (mmol/L)	4.10 - 5.60	5.10 (4.65-5.40)	5.90 (5.40-6.20)	**0.0001**
2 hr OGTT (mmol/L)	<7.80	5.85 (4.85-7.00)	9.25 (8.40-11.00)	**< 0.0001**
HbA1c (%)	4.50-6.30	5.75 (5.40-6.05)	6.30 (6.00-6.60)	**< 0.0001**
Insulin Fasting (mIU/L)	0.20-9.40	6.55 (4.10-9.35)	9.95 (7.20-16.30)	**0.0092**
Triglycerides (mmol/L)	1.80-2.20	1.13 (0.95-1.56)	1.63 (1.28-2.36)	0.1368
LDL Cholesterol (mmol/L)	1.00-3.00	3.65 (3.20-4.55)	3.95 (3.20-4.50)	0.9586
HDL Cholesterol (mmol/L)	1.00-1.50	1.20 (1.10-1.50)	1.40 (1.10-1.50)	0.6398
Ultra-sensitive CRP (mg/L)	1.00-3.00	4.84 (2.49-6.83)	8.72 (3.37-18.36)	0.1764

Significant values (p<0.05) are in bold text, Median [IQR].

4.3 Haematological parameters

There was no significant difference in the haematological parameters between the hyperglycaemic and control group (table 4.3).

Table 4.3: Haematological parameters

Full blood counts	Reference range	Median (25Q-75Q)		P=value
		Normal N35	Hyperglycaemia N34	
Red Cell Count (x10^{12}/L)	4.5-5.5 x10^{12}/L	4.61 (4.19-4.90)	4.76 (4.54-5.01)	0.2617
Haematocrit (L/L)	40.0-50.0 %	0.41 (0.38-0.43)	0.41 (0.41-0.44)	0.2210
Haemoglobin (g/dL)	13.0-17.0 g/dL	12.9 (12.4-14.0)	13.6 (12.9-14.3)	0.2056
MCV (fl)	79.1-98.9 fl	88.0 (83.0-92.0)	87.5 (84.0-92.0)	0.8296
MCH (pg)	27.0-32.0 pg	29.0 (26.0-30.0)	28.0 (27.0-30.0)	0.7982
MCHC (g/dL)	32.0-36.0 g/dL	32.0 (32.0-33.0)	33.0 (32.0-33.0)	0.7049
RDW (%)	11.6-14.0 %	14.4 (13.5-15.0)	14.2 (13.5-15.0)	0.3762
White Cell Count (x10^9/L)	4.0-10.0 x10^9/L	6.60 (5.60-7.90)	7.50 (6.50-9.40)	0.1444
Lymphocytes %	20-40 %	30.7 (25.5-40.0)	31.5 (25.4-39.0)	0.9024
Lymphocytes ABS (x10^9/L)	1.0-3.0 x10^9/L	2.20 (1.80-2.55)	2.35 (1.90-3.00)	0.1107
Monocytes %	2.0-10.0 %	6.05 (5.05-7.60)	5.30 (4.50-7.10)	0.1886
Monocytes ABS (x10^9/L)	0.2-1.0 x10^9/L	0.40 (0.36-0.50)	0.40 (0.37-0.50)	0.6968
Neutrophils %	40-80 %	58.9 (50.2-65.6)	59.3 (51.8-68.0)	0.7301
Neutrophils ABS (x10^9/L)	2.0-7.0 x10^9/L	3.95 (2.85-4.90)	4.75 (3.30-6.00)	0.2342
Basophils %	<1.0-2.0 %	0.40 (0.30-0.50)	0.40 (0.30-0.50)	0.5576
Basophils ABS (x10^9/L)	0.02-.01 x10^9/L	0.01 (0.01-0.01)	0.01 (0.01-0.01)	0.1361
Eosinophils %	1.0-6.0 %	2.15 (1.55-3.80)	1.65 (1.20-2.80)	0.2642
Eosinophils ABS (x10^9/L)	0.02-0.5 x10^9/L	0.20 (0.10-0.20)	0.10 (0.10-0.20)	0.6033
Platelet Count (x10^9/L)	137-373 x10^9/L	264 (229-308)	259 (226-327)	0.9808

4.4 T-cell antigen expression in normoglycaemia and hyperglycaemia participants

The percentage (%) of T-cells expressing CD95, CD38 and HLA-DR was not significantly different in the hyperglycaemic group when compared to the normoglycaemic group pre glucose incubation. The same pattern was observed when the mean fluorescent intensity (MFI) was compared between the two groups. There was also no significant difference between the % of T-cells expressing PD-1 between the two groups, p=0.8837. The same trend was observed when comparing the MFI, p=0.5904 (see table 4.4).

Table 4.4: Pre incubation T-cell marker expression in hyperglycaemia and normoglycaemia

%	Median (Q25-75)		P	MFI	Median (Q25-75)		P
	Normal	HG			Normal	HG	
CD3				**CD3**			
$CD3^+$ $\%CD95^+$	30.17 (25.62-36.37) N35	28.67 (25.73-35.33) N34	0.7225	$CD3^+$ $MFICD95^+$	3.25 (3.13-3.34) N35	3.20 (3.10-3.27) N35	0.2615
$CD3^+$ $\%HLA-DR^+$	20.99 (16.29-26.32) N24	17.81 (13.65-24.30) N26	0.3570	$CD3^+$ $MFIHLA-DR^+$	2.09 (1.92-2.32) N24	2.22 (1.94-3.19) N26	0.9245
$CD3^+$ $\%CD38^+$	10.57 (7.60-22.22) N3	17.95 (11.80-23.34) N7	0.4337	$CD3^+$ $MFICD38^+$	5.06 (2.82-5.27) N3	2.97 (2.61-3.15) N7	0.4899
$CD3^+$ $\%PD1^+$	22.09 (16.99-26.05) N26	19.85 (15.97-25.72) N27	0.8837	$CD3^+$ $MFIPD1^+$	18.32 (17.38-19.79) N26	17.91 (15.82-20.45) N28	0.5904
CD4				**CD4**			
$CD4^+$ $\%CD95^+$	50.18 (45.29-54.70) N35	50.75 (43.93-56.60) N34	0.9282	$CD4^+$ $MFICD95^+$	3.06 (2.93-3.15) N35	2.98 (2.89-3.12) N35	0.2578
$CD4^+$ $\%HLA-DR^+$	11.04 (7.80-14.77) N24	11.53 (8.36-16.31) N26	0.9393	$CD4^+$ $MFIHLA-DR^+$	2.99 (2.70-4.92) N24	3.17 (2.69-4.26) N25	0.3348
$CD4^+$ $\%CD38^+$	25.25 (18.25-32.25) N2	29.44 (15.13-38.14) N7	0.8261	$CD4^+$ $MFICD38^+$	3.48 (3.29-3.66) N2	3.18 (2.75-4.44) N7	0.8897
$CD4^+$ $\%PD1^+$	19.05 (16.82-23.91) N26	20.96 (16.53-25.87) N27	0.5196	$CD4^+$ $MFIPD1^+$	18.81 (17.10-20.70) N26	18.97 (17.33-20.82) N28	0.8095
CD8				**CD8**			
$CD8^+$ $\%CD95^+$	25.27 (14.95-32.12) N35	22.51 (18.49-30.93) N34	0.8268	$CD8^+$ $MFICD95^+$	2.50 (2.43-2.60) N35	2.49 (2.37-2.56) N34	0.4691
$CD8^+$ $\%HLA-DR^+$	29.21 (21.80-34.55) N23	25.40 (16.37-37.25) N24	0.4776	$CD8^+$ $MFIHLA-DR^+$	2.16 (2.00-2.51) N23	2.24 (2.01-2.60) N23	0.3636
$CD8^+$ $\%CD38^+$	17.03 (17.03-17.03) N1	26.50 (25.27-42.51) N5	0.4383	$CD8^+$ $MFICD38^+$	2.64 (2.64-2.64) N1	4.34 (3.25-4.74) N5	0.2130

HG- Hyperglycaemia

4.5 The effects of glucose on T-cell antigen expression

To investigate the effects of *ex vivo* hyperglycaemic conditions on antigen expression, 31 (16 hyperglycaemic and 15 normoglycaemic individuals) of the 69 participants were randomly selected and their T-cells incubated with 30mmol/L glucose concentration for 45 minutes.

4.5.1 Fas (CD95)

Although not statistically significant, the MFI and % of $CD3^+$ T-cells expressing CD95 after exposure to glucose was decreased in both hyperglycaemic and normoglycaemic groups. There was however a significantly decreased percentage of $CD4^+$ T-cells expressing CD95 after incubation with glucose in the normoglycaemic group (p=0.0213) and while the MFI was also lower after incubation this was not statistically significant. A similar pattern was observed in the hyperglycaemic group of participants, in which a significantly reduced percentage of $CD4^+$ T-cells expressed CD95 in hyperglycaemic conditions (p=0.0349) (See Fig 4.1 and Table 4.5).

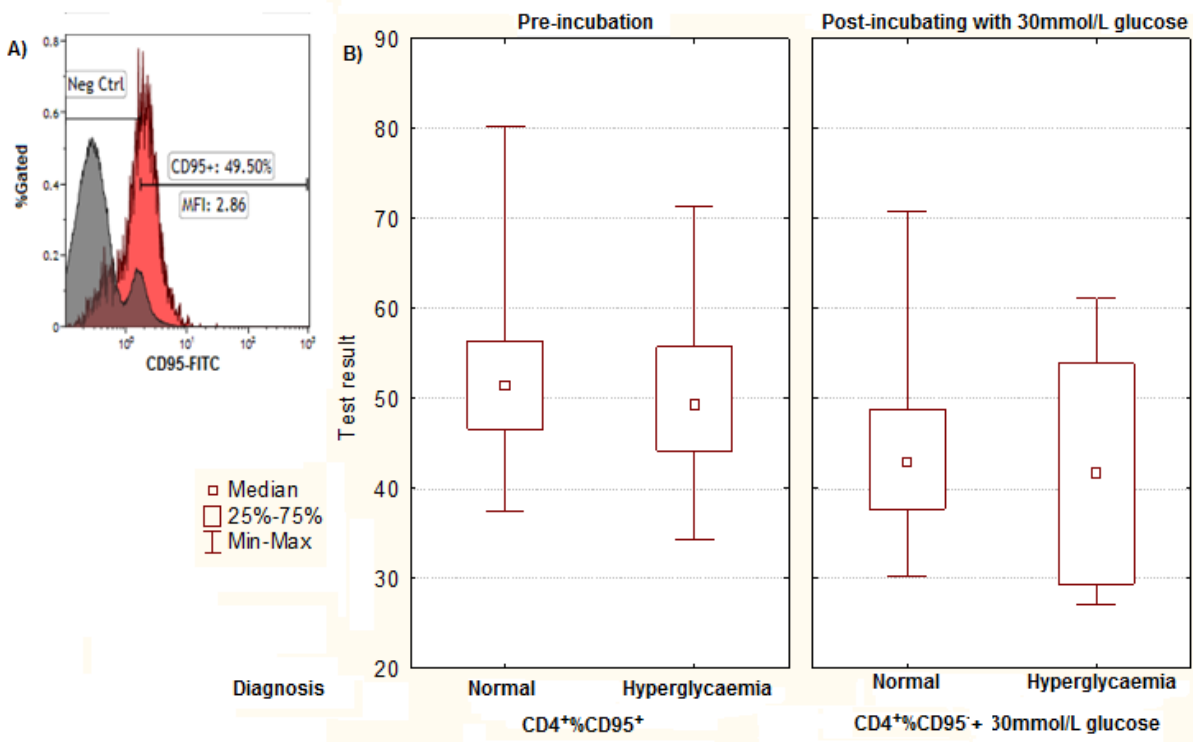

Figure 4.1: The percentage of $CD4^+$ T-cells expressing CD95 in normoglycaemia and hyperglycaemia. **Plot A** shows how the percentage expression and MFI of CD95 was determined. An FMO was used as a negative control to distinguish positive from negative evens. **B)** The median % of $CD4^+$ T-cells expressing CD95 was significantly decreased in both normoglycaemic and hyperglycaemic group after incubating with 30 mmol/L glucose, p=values 0.0213 and 0.0349 respectively.

Table 4.5: CD95 expression on T-cells pre and post glucose incubation

Median IQR	Normoglycaemia		P
	Pre incubation	**Post incubation**	
$CD3^+$%$CD95^+$	31.8(26.9-40.4)	26.5(21.3-32.1)	0.0967
$CD4^+$%$CD95^+$	51.35(46.56-56.34)	42.82(37.64-48.80)	**0.0213**
$CD8^+$%$CD95^+$	27.1(17.1-33.4)	21.4(14.7-26.6)	0.2186
$CD3^+$MFI$CD95^+$	3.27(3.20-3.33)	3.17(3.09-3.24)	0.0620
$CD4^+$MFI$CD95^+$	3.06(2.96-3.11)	2.96(2.85-3.01)	0.0924
$CD8^+$MFI$CD95^+$	2.50(2.44-2.61)	2.48(2.41-2.59)	0.5867
	Hyperglycaemia		
	Pre incubation	**Post incubation**	
$CD3^+$%$CD95^+$	29.9(26.3-34.0)	23.3(18.6-32.4)	0.1103
$CD4^+$%$CD95^+$	49.27(44.13-55.76)	41.73(29.34-53.84)	**0.0349**
$CD8^+$%$CD95^+$	24.0(19.0-31.9)	18.1(13.7-28.3)	0.2556
$CD3^+$MFI$CD95^+$	3.14(3.09-3.25)	3.09(2.99-3.17)	0.1080
$CD4^+$MFI$CD95^+$	2.96(2.84-3.06)	2.84(2.73-2.97)	0.1242
$CD8^+$MFI$CD95^+$	2.49(2.39-2.56)	2.45(2.38-2.52)	0.4690

Significant values ($p<0.05$) are in bold text, median [IQR].

4.5.2 HLA-DR

Although after incubation with glucose, the percentage and MFI of T-cells expressing HLA-DR was lower this was not statistically significant in all T-cell subsets (see table 4.6). Analysis of the 31 patients in these experiments did however demonstrate a significant difference in the MFI of HLA-DR expression between the normoglycaemic and hyperglycaemic on $CD4^+$ T-cells, p=0.0252. This further decreased after incubation with glucose but the difference was not significant (see Fig 4.2).

Table 4.6: HLA-DR expression on T-cells pre and post glucose incubation

Median IQR	Normoglycaemia		P
	Pre incubation	Post incubation	
$CD3^+$%$HLA\text{-}DR^+$	19.6(17.3-22.9)	13.7(13.7-20.8)	0.5355
$CD4^+$% $HLA\text{-}DR^+$	11.04(10.11-13.54)	8.92(6.22-11.67)	0.5332
$CD8^+$% $HLA\text{-}DR^+$	21.9(15.9-28.4)	17.9(11.7-25.3)	0.6063
$CD3^+$MFI $HLA\text{-}DR^+$	6.77(3.26-6.92)	6.39(5.85-7.12)	0.4036
$CD4^+$MFI $HLA\text{-}DR^+$	7.44(7.25-7.98)	6.62(5.58-7.68)	0.6790
$CD8^+$MFI $HLA\text{-}DR^+$	2.75(2.36-3.28)	2.94(2.44-3.34)	0.8458
	Hyperglycaemia		
	Pre incubation	**Post incubation**	
$CD3^+$% $HLA\text{-}DR^+$	11.7(7.9-17.3)	11.2(6.0-13.2)	0.1678
$CD4^+$% $HLA\text{-}DR^+$	8.75(6.02-14.45)	7.13(4.42-9.53)	0.2540
$CD8^+$% $HLA\text{-}DR^+$	16.7(13.2-28.3)	20.9(16.6-26.0)	0.9951
$CD3^+$MFI $HLA\text{-}DR^+$	4.09(3.37-5.17)	3.55(3.15-5.28)	0.8798
$CD4^+$MFI $HLA\text{-}DR^+$	4.20(2.39-5.34)	4.04(2.63-6.08)	0.1494
$CD8^+$MFI $HLA\text{-}DR^+$	2.76(2.60-3.01)	2.67(2.49-2.74)	0.7036

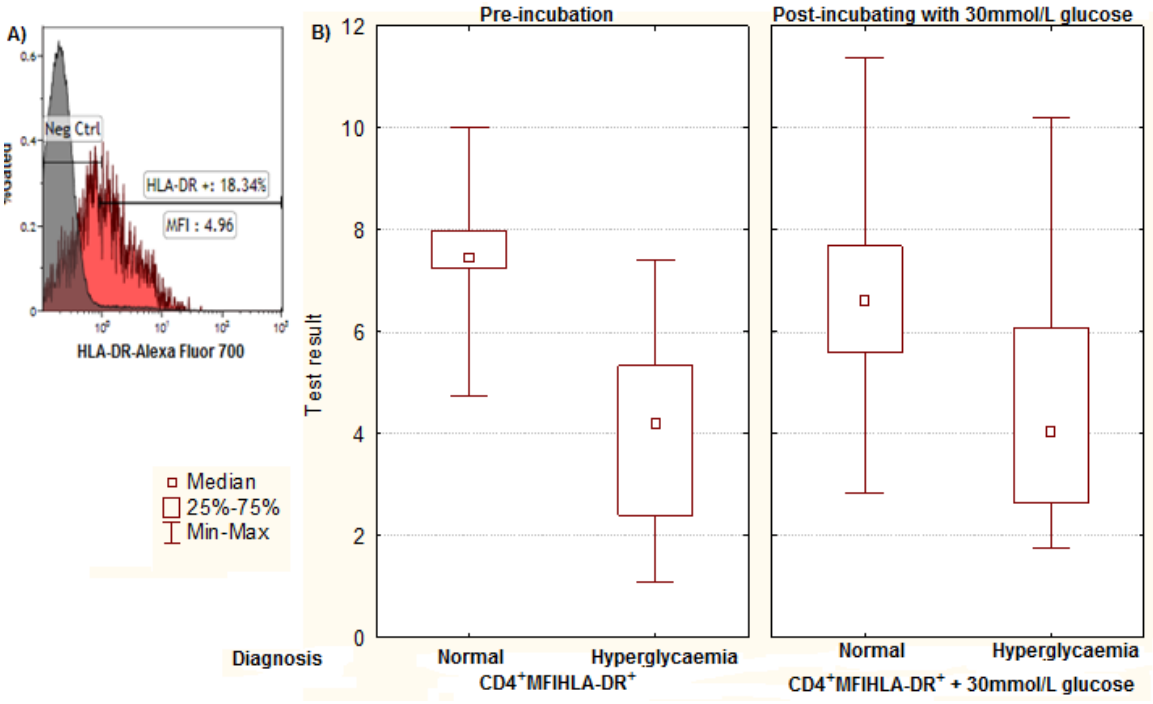

Figure 4.2: The MFI of HLA-DR on $CD4^+$ T-cells in normoglycaemic and hyperglycaemic groups. **Plot A** illustrates the gating strategy of HLA-DR. **B)** At baseline, the HLA-DR median was significantly lower in the hyperglycaemic group 4.20(2.39-5.34) compared to the normoglycaemic group 7.44(7.25-7.98), p=value 0.0252. Incubation with glucose further decreased HLA-DR expression although the P-values were insignificant.

4.5.3 PD-1

There was no significant difference in the expression of PD-1 post incubation with glucose. This pattern was similar in both groups of participants. (See table 4.7 and Fig 4.3).

Table 4.7: PD-1 expression on T-cells pre and post glucose incubation

Median IQR	Normoglycaemia		P
	Pre incubation	**Post incubation**	
$CD3^+$%$PD\text{-}1^+$	17.8(13.2-22.1)	16.2(13.4-17.3)	0.5574
$CD4^+$% $PD\text{-}1^+$	16.1(10.1-20.7)	15.3(10.5-19.2)	0.6143
$CD3^+$MFI $PD\text{-}1^+$	16.2(15.9-17.9)	16.2(15.2-16.9)	0.4900
$CD4^+$MFI $PD\text{-}1^+$	17.0(16.2-18.2)	17.3(16.8-17.8)	0.9283
	Hyperglycaemia		
	Pre incubation	**Post incubation**	
$CD3^+$% $PD\text{-}1^+$	19.4(16.3-22.3)	18.2(16.5-22.4)	0.2209
$CD4^+$% $PD\text{-}1^+$	18.3(14.6-21.0)	16.7(14.2-19.8)	0.2047
$CD3^+$MFI $PD\text{-}1^+$	16.0(15.4-17.4)	15.9(15.4-16.8)	0.8773
$CD4^+$MFI $PD\text{-}1^+$	17.8(16.3-19.5)	17.4 (16.0-17.7)	0.7608

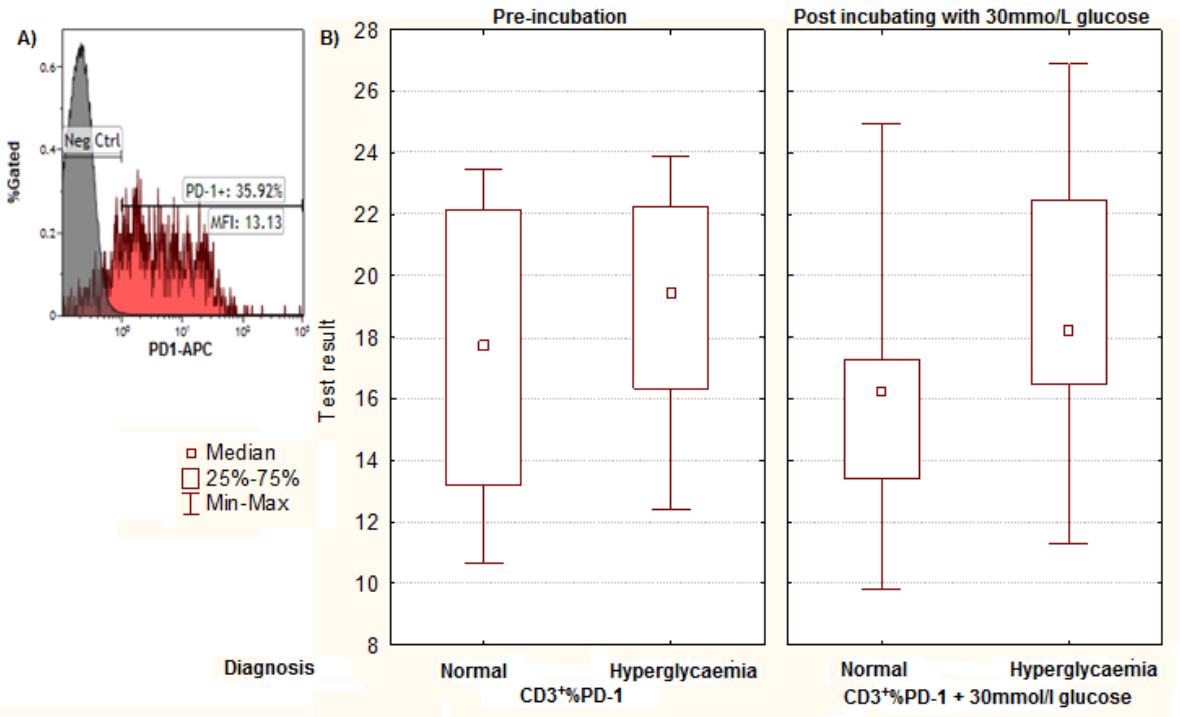

Figure 4.3: PD-1 expression on $CD3^+$ T-cells in normoglycaemia and hyperglycaemia. **Plot A** illustrates PD-1 gating strategy. **B)** The median % expression of PD-1 was not significantly increased in the hyperglycaemic group compared to the normoglycaemic group and there was no significant difference after incubation with glucose.

4.5.4 CD38

Ten (10) samples were processed for the expression of CD38 of which 3 were normoglycaemic and 7 were hyperglycaemic. Incubation with 30mmol/L glucose resulted in a decreased % of $CD3^+$ T-cells expressing CD38. A similar pattern was observed when examining the MFI. These results were similar in both the $CD4^+$ and $CD8^+$ subsets. Furthermore, the hyperglycaemic group had lower percentages of all T-cell subsets expressing the antigen when compared to the normoglycaemic group in both the pre- and post-incubation analysis. Due to the low numbers of samples in this group statistical analysis could not be performed.

4.6 T-cell antigen expression in hypoglycaemic and hyperglycaemic conditions

The T-cells of 31 participants were incubated with increasing concentrations of glucose (2mmol/l, 5mmol/l and 30mmol/l) mimicking hypoglycaemia, normoglycaemia and hyperglycaemia respectively (Oleszczak et al., 2012). Incubation with glucose showed a general decrease in the median % of cells expressing CD95 and MFI. These results further confirmed that the % of $CD4^+$ T-cells expressing CD95 significantly decreased with increasing concentrations of glucose in both the normo and hyperglycaemic groups (see Fig 4.4 and table 4.8). There was however no significant differences when the $CD3^+$ and $CD8^+$ subsets were analysed.

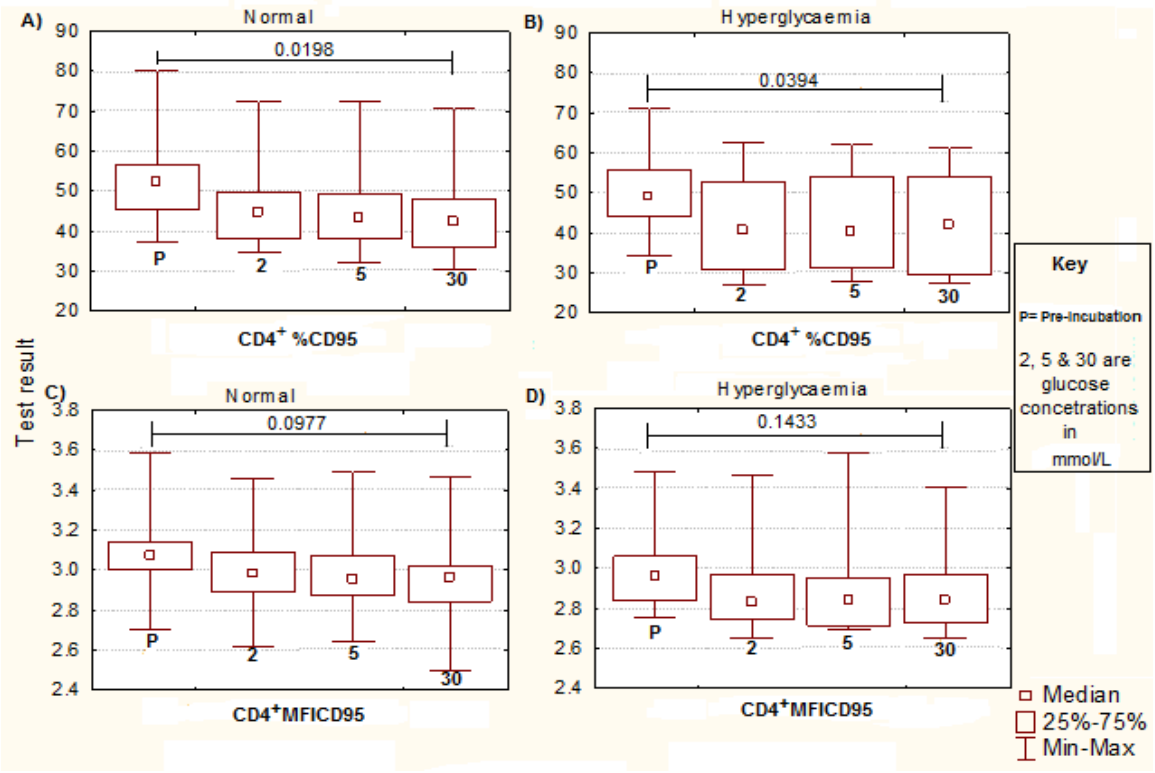

Figure 4.4: CD95 expression in varying concentrations of glucose. **Plot A** and **Plot B** illustrates the % of $CD4^+$ T-cells expressing CD95 in the control group and test group at pre-incubation and post-incubation in 2 mmol/L, 5 mmol/L and 30mmol/L glucose concentrations. **Plot C & D** illustrates the MFI of CD95 on CD4 T-cells in control and test group, respectively.

Table 4.8: Changes in T-cell antigen expression with increasing glucose concentration in both groups.

Parameters	**P-values**						
	{2}	**{3}**	**{4}**	**{5}**	**{6}**	**{7}**	**{8}**
$CD4^+$ %$CD95^+$ **Normal {1}**	0.477	0.0716	0.0058	**0.0359**	0.0081	**0.0198**	0.0067
$CD4^+$ %$CD95^+$ **Hyperglycaemia {2}**		0.2592	**0.0351**	0.1515	**0.0466**	0.0941	**0.0394**
$CD4^+$ %$CD95^+$ 2mmol **Normal {3}**			0.3371	0.7610	0.3997	0.5865	0.3614
$CD4^+$ %$CD95^+$ 2mmol **Hyperglycaemia {4}**				0.5143	0.9042	0.6828	0.9614
$CD4^+$ %$CD95^+$ 5mmol **Normal {5}**					0.5934	0.8103	0.5454
$CD4^+$ %$CD95^+$ 5mmol **Hyperglycaemia {6}**						0.7715	0.9426
$CD4^+$%$CD95^+$ 30 mmol **Normal {7}**							0.7180
$CD4^+$%$CD95^+$ 30mmol **Hyperglycaemia {8}**							

Significant values ($p<0.05$) are in bold text. For example, the difference the expression of CD95 expression on $CD4^+$ T-cells in the hyperglycaemic group at baseline {2} and 2mmol/l {4}, 5mmol/l {6} and 30mmol/l {8} glucose concentration was significant, p=0.0351, p=0.0466 and 0.0394 respectively.

4.7 Correlations between T-cell markers, glucose metabolism and inflammation

There were no significant correlations between T-cell antigens expression, fasting glucose, 2hr OGTT and HbA1c. However, there was a significant correlation between the inflammatory marker CRP and the % of $CD3^+$ T cells expressing CD95, p=0.0242. The same correlation was seen with both $CD4^+$ and $CD8^+$ T-cells expressing CD95, p=0.0073 and 0.0087 respectively. The MFI of $CD4^+$ T-cells expressing HLA-DR also showed significant correlation with CRP marker, p=0.0362. Furthermore, there was a significant correlation between insulin fasting and % of $CD3^+$ and $CD8^+$ T-cells expressing CD38, p=0.0158 and 0.0048. The same insulin fasting correlation was seen with $CD4^+$ T-cells expressing PD-1, p=0.0313 (see table 4.9, 4.10 and 4.11).

Table 4.9: Correlation between CD3 marker, glucose metabolism and inflammation.

CD3 Marker	Variables	N	Results in %		Results in MFI	
			R	P	R	P
$CD3^+CD95^+$	**Fasting Glucose** (mmol/L)	69	-0.0842	0.4916	-0.0622	0.6119
	2 hr OGTT (mmol/L)	69	0.1130	0.3554	-0.1591	0.1916
	HbA1c (%)	69	0.0608	0.6195	-0.1908	0.1163
	Insulin Fasting (mIU/L)	69	-0.0884	0.4701	-0.0857	0.4838
	Ultra-sensitive CRP (mg/L)	69	0.2712	**0.0242**	0.2292	0.0582
$CD3^+HLA\text{-}DR^+$	**Fasting Glucose** (mmol/L)	50	-0.0741	0.6090	-0.0458	0.7524
	2 hr OGTT (mmol/L)	50	-0.1456	0.3130	0.1871	0.1933
	HbA1c (%)	50	-0.1356	0.3476	-0.1316	0.3623
	Insulin Fasting (mIU/L)	50	0.0444	0.7596	-0.0562	0.6984
	Ultra-sensitive CRP (mg/L)	50	0.1427	0.3228	-0.1558	0.2801
$CD3^+PD1^+$	**Fasting Glucose** (mmol/L)	53	-0.0286	0.8391	0.0562	0.6863
	2 hr OGTT (mmol/L)	53	-0.0790	0.5740	-0.0521	0.7085
	HbA1c (%)	53	-0.0905	0.5191	0.0165	0.9056
	Insulin Fasting (mIU/L)	53	0.1468	0.2942	-0.0278	0.8419
	Ultra-sensitive CRP (mg/L)	53	0.1737	0.2136	0.0148	0.9152
$CD3^+CD38^+$	**Fasting Glucose** (mmol/L)	10	0.2675	0.4550	-0.1945	0.5902
	2 hr OGTT (mmol/L)	10	0.2121	0.5563	-0.5394	0.1076
	HbA1c (%)	10	0.4377	0.2058	-0.3891	0.2665
	Insulin Fasting (mIU/L)	10	0.7333	**0.0158**	0.0667	0.8548
	Ultra-sensitive CRP (mg/L)	10	0.1216	0.7379	0.3951	0.2584

Significant values ($p<0.05$) are in bold text

Table 4.10: Correlation between CD4 marker, glucose metabolism and inflammation.

CD4 Marker	Variables	N	Results in %		Results in MFI	
			R	P	R	P
$CD4^+CD95^+$	**Fasting Glucose** (mmol/L)	69	-0.0119	0.9227	-0.0623	0.6111
	2 hr OGTT (mmol/L)	69	0.0338	0.7831	-0.1742	0.1522
	HbA1c (%)	69	0.0904	0.4601	-0.2223	0.0664
	Insulin Fasting (mIU/L)	69	-0.0404	0.7414	-0.1542	0.2059
	Ultra-sensitive CRP (mg/L)	69	0.3203	**0.0073**	0.1713	0.1593
$CD4^+HLA\text{-}DR^+$	**Fasting Glucose** (mmol/L)	50	-0.0041	0.9775	-0.0995	0.4962
	2 hr OGTT (mmol/L)	50	0.0445	0.7589	-0.0376	0.7975
	HbA1c (%)	50	-0.0168	0.9078	-0.2595	0.0718
	Insulin Fasting (mIU/L)	50	0.2616	0.0665	-0.2661	0.0646
	Ultra-sensitive CRP (mg/L)	50	0.1545	0.2841	-0.3000	**0.0362**
$CD4^+PD1^+$	**Fasting Glucose** (mmol/L)	54	0.1436	0.3051	0.1535	0.2679
	2 hr OGTT (mmol/L)	54	-0.0016	0.9911	-0.0177	0.8992
	HbA1c (%)	54	0.1026	0.4646	-0.0941	0.4983
	Insulin Fasting (mIU/L)	54	0.2962	**0.0313**	-0.0032	0.9818
	Ultra-sensitive CRP (mg/L)	54	0.2269	0.1023	-0.0030	0.9828
$CD4^+CD38^+$	**Fasting Glucose** (mmol/L)	9	0.0502	0.8979	-0.1590	0.6828
	2 hr OGTT (mmol/L)	9	-0.0167	0.9661	-0.3667	0.3317
	HbA1c (%)	9	0.0333	0.9322	-0.1167	0.7650
	Insulin Fasting (mIU/L)	9	0.6000	0.0876	0.2500	0.5165
	Ultra-sensitive CRP (mg/L)	9	0.4167	0.2646	0.6333	0.0671

Significant values ($p<0.05$) are in bold text

Table 4.11: Correlation between CD8 marker, glucose metabolism and inflammation.

CD8 Marker	Variables	N	Results in %		Results in MFI	
			R	P	R	P
$CD8^+CD95^+$	**Fasting Glucose** (mmol/L)	69	-0.0208	0.8650	-0.0657	0.5917
	2 hr OGTT (mmol/L)	69	0.1553	0.2025	-0.0134	0.9133
	HbA1c (%)	69	0.0147	0.9048	-0.0536	0.6621
	Insulin Fasting (mIU/L)	69	-0.1463	0.2303	0.0298	0.8077
	Ultra-sensitive CRP (mg/L)	69	0.1378	0.2589	0.3094	**0.0097**
$CD8^+HLA\text{-}DR^+$	**Fasting Glucose** (mmol/L)	47	0.0166	0.9120	-0.0547	0.7179
	2 hr OGTT (mmol/L)	47	-0.0068	0.9637	0.2390	0.1097
	HbA1c (%)	47	-0.0140	0.9257	0.0002	0.9990
	Insulin Fasting (mIU/L)	47	0.1019	0.4953	-0.1636	0.2774
	Ultra-sensitive CRP (mg/L)	47	0.1583	0.2879	-0.1131	0.4544
$CD8^+CD38^+$	**Fasting Glucose** (mmol/L)	6	0.5798	0.2278	0.0580	0.9131
	2 hr OGTT (mmol/L)	6	0.2571	0.6228	0.1429	0.7872
	HbA1c (%)	6	0.7714	0.0724	-0.0857	0.8717
	Insulin Fasting (mIU/L)	6	0.9429	**0.0048**	-0.2571	0.6228
	Ultra-sensitive CRP (mg/L)	6	0.5429	0.2657	0.4857	0.3287

Significant values ($p<0.05$) are in bold text

CHAPTER FIVE

DISCUSSION

The aim of this current study was to investigate the expression of T-cell activation and exhaustion markers in hyperglycaemic individuals (pre-diabetics and T2DM) and to compare the results to a cohort of normoglycaemic participants. In addition, we aimed to investigate the effects of increased glucose concentrations on T-cell antigen expression and further correlate the expression of these activation and exhaustion markers with glucose metabolism and routine biochemical markers.

The results demonstrated no significant difference in the expression of T-cell activation antigens between the hyperglycaemic and normoglycaemic groups pre incubation with glucose. However, in the subgroup of participants whose T-cells were incubated with glucose, there was a significant difference in the mean florescence intensity of HLA-DR on $CD4^+$ T-cells between the normoglycaemic and hyperglycaemic group at baseline. In addition, after incubation with high concentrations of glucose, the percentage of $CD4^+$ T-cells expressing CD95 decreased significantly in both groups.

These results differ from other reports in the literature which have demonstrated significant differences between normoglycaemic and hyperglycaemic individuals. Examples of these include a study conducted on one hundred and eight (108) newly diagnosed and known diabetics with myocardial infarction. Thirty six (36) of the patients were known cases of DM whilst 31 were newly diagnosed cases of hyperglycaemia with glucose levels of >7mmol/l. Using flow cytometry to analyse surface HLA-DR expression and automated turbidimetry to measure CRP, the results demonstrated significantly increased % expression of HLA-DR on both $CD4^+$ and $CD8^+$ T-cells in the hyperglycaemic group compared to those with normoglycaemia. They further reported significantly increased CRP levels in hyperglycaemic patients (Marfella et al., 2003).

Our findings differ from this study. This could be attributed to the fact that the diagnosis of hyperglycaemia in this study was based solely on random glucose levels and the diabetic group consisted of both T1DM and T2DM. Furthermore and importantly, the study was performed on patients who presented with myocardial infarction which has been associated with chronic inflammation and immune activation. Cytokines secreted by activated immune cells result in the vascular endothelial surface attracting both platelets and leucocytes which play a major role in the development of plaques and cardiovascular disease (CVDs) (Rondina et al., 2013). In this current study, none of the participants had cardiovascular disease and this important difference could account for the dissimilar results. These findings could further imply that the immune activation observed in diabetic individuals maybe

associated with the acceleration of the disease and the development of complications such as CVDs.

Researches investigating the expression of PD-1 on the surface of T-cells have also demonstrated increased positivity in association with diabetic complications such atherosclerosis, sepsis and retinopathy (Shi et al., 2013; Fang et al., 2015; Jia et al., 2016). Jia et al investigated the cell surface expression of PD-1, in 245 patients. Of these, 80 were known cases of T2DM without infection, 77 were known T2DM with severe sepsis and 88 were non-diabetic patients with severe sepsis. The results were compared to 50 healthy controls. The authors reported significantly increased percentage of $CD4^+$ and $CD8^+$ T-cells expressing PD1 together with high levels of C-reactive protein (CRP) (Jia et al., 2016). These results were similar to another study of 125 T2DM patients in which significantly increased PD-1 expression on $CD4^+$ T-cells was reported. In this investigation, 48 of the DM patients had atherosclerotic macrovascular complications and 25 had acute coronary syndrome (Shi et al., 2013). A further investigation examined PD-1 expression in 79 patients with proliferative diabetic retinopathy and compared the results to 60 without proliferative retinopathy and 93 normal controls. The patients with retinopathy once again had significantly increased expression of PD-1 compared to those who did not have this complication (Fang et al., 2015). The above studies strongly suggest that the increased expression of activation and exhaustion antigens observed on the surface of T-cells is associated with the development of diabetic complications. These observations could explain the differences observed in the current study which excluded long term diabetics with complications.

PD-1 is an immunoreceptor that inhibits T-cell function by delivering a negative co-stimulatory signal upon interacting with its ligands (Jin et al., 2011; Riella et al., 2012). Resting T-cells do not express PD-1 but it is upregulated on activated T-cells (Chinai et al., 2015) and is indicative of on-going T-cell activation (Okazaki & Honjo, 2007; Liechtenstein et al., 2012). The up-regulation of PD-1 on T-cells induces a hypo-responsive state termed exhaustion or anergy (Xing & Hogquist, 2012). These findings suggest that hyperglycaemia could induce T-cell exhaustion by increasing PD-1 expression. This may lead to loss of their effector function and the ability to mount an effective immune response predisposing individuals with T2DM to increased infections and sepsis.

Obesity is a major cause of chronic inflammation which results in the activation of T-cells and increased T-cell antigen expression. This phenomenon could provide a further explanation for the fact that there was no significant difference in the expression of T-cell activation antigens between the normoglycaemic and hyperglycaemic participants. In this current study

both the normoglycaemic and hyperglycaemic participants had increased BMI's (>26), with the majority of the participants fulfilling the criteria of obesity (BMI=>30). This theory is further supported by the high levels of Ultra-sensitive CRP, a marker of inflammation, in both groups. Obesity is an important cause of chronic inflammation and is initiated by adipose tissue adipocytes becoming hypertrophic in an effort to accommodate excess triglyceride storage (Chng et al., 2015; Ip et al., 2015). The adipocytes eventually undergo apoptotic death which triggers the production of pro-inflammatory cytokines and adipokines. This continual process leads to chronic inflammation with the consequent activation of T-cells and other cells of the immune system (Lontchi-Yimagou et al., 2013). The results from this current study imply that obesity as well as hyperglycaemia could be an important driver in the chronic inflammation observed in these individuals.

A further explanation for the dissimilar results could be the differing population groups and methodologies used to analyse the expression of T-cell antigens. In the current study, participants with pre-diabetes and newly diagnosed diabetics were grouped together as hyperglycaemia, whereas the other reports have included long term diabetic patients. As the activation of both the innate and adaptive immune responses could vary across the different glucose tolerance groups, it is important to increase participant numbers in order to study and compare each subgroup individually. In addition our study has attempted to analyse specific T-cell subsets as opposed to analysing all lymphocytes.

However, although the majority of reports differ from ours, similar findings were reported by another flow cytometry based study. The authors of this article demonstrated no significant differences between the expression of CD95 on the T-cells in T2DM and the control group. A total of 30 patients were included of which 22 had T1DM, 8 had T2DM and 12 were controls. The patients were classified according the National Diabetes Data Group criteria (Giordano et al., 1995).

In order to further investigate the effect of hyperglycaemia on T-cell antigen expression, we incubated the lymphocytes of 32 of the 69 participants, with increased concentrations of glucose. The results of this experiment demonstrated a significantly decreased surface expression of HLA-DR and CD95 on the $CD4^+$ T-cell subset in both normoglycaemic and hyperglycaemic individuals. These results were further supported by incubating the T-cells with increasing doses of glucose which resulted in a steady decrease in CD95 expression on the $CD4^+$ subset but not on $CD8^+$ T-cells. These observations seem to imply that high concentrations of glucose inhibit $CD4^+$ T-cell activation by decreasing the expression of T-cell activation markers which could possibly induce a state of immune exhaustion.

CD95 (Fas) has been shown to play an important role in the transport of glucose into the cells and also in the initiation of activation-induced cell death (AICD) (Berridge et al., 1996). Previous research has reported that the activation of the CD95 signalling pathway inhibits glucose uptake by reducing the affinity of the glucose transporter 1 (GLUT1) for glucose (Berridge et al., 1996). This therefore suggests that there is a strong relationship between CD95 signalling and glucose transportation. To our knowledge, the exact effects of decreased expression of CD95 on glucose transport and transport function is still unknown. More studies still need to be done to investigate this phenomenon. However, a dysregulation of CD95 expression could result in abnormal and deregulated glucose transportation into cells and might contribute to the hyperglycaemia observed in T2DM.

The $CD4^+$ T-helper cells play an important role in the adaptive immune response and evidence suggests that this subset could play an important role in the pathogenesis of T2DM especially in the initiation of low grade chronic inflammation (Xia et al., 2017). The role of $CD4^+$ T-cells in obesity and insulin resistance has been well described with the former being able to induce MHC class II expression on adipocytes, thus activating the $CD4^+$ cells and initiating tissue inflammation (Deng, et al, 2013). This triggers the $CD4^+$ effector T-cells pro-inflammatory subsets (Th1 and Th17) to release cytokines that further exacerbate inflammation. The balance between pro-inflammatory and anti-inflammatory (Th2 and T_{regs}) subsets is important in immune regulation and control of the inflammatory process. In T2DM, the $CD4^+$ T-cells' anti-inflammatory/pro-inflammatory ratio was reported to be decreased when compared to the normoglycaemic group (Zeng et al., 2012). Further evidence suggests that T_{reg} cells are decreased in T2DM (Jagannathan-bogdan et al., 2011). Thus, these studies all seem to suggest that hyperglycaemia may induce immune dysregulation and dysfunction mediated by T-helper cells. The results of this current study seem to support this theory. In addition, the HLA-DR (MHC class II) signalling via antigen presenting cells (APCs) and $CD4^+$ T-cells is important in initiating an adaptive immune response (Costantino et al., 2009). Therefore, reduced expression of HLA-DR could further result in a dysfunctional adaptive immune response.

Our results showed no correlations between T-cell antigen expression and glucose metabolism. However, there was a significant correlation between the ultra-sensitive CRP and the expression of HLA-DR on $CD4^+$ T-cells and CD95 on all T-cell subsets. C-reactive protein (CRP) is a sensitive marker of low-grade systemic inflammation that is synthesised by hepatocytes in response to pro-inflammatory cytokines such as IL-6 (Shrivastava et al., 2015). In T2DM, CRP has been shown to directly promote insulin resistance and also stimulate the over production of adhesion molecules such as selectins which are critical mediators of endothelial dysfunction in CVDs (Hu et al., 2004; Reyes et al., 2015). A study

examining inflammatory markers in T2DM reported increased CRP levels compared to normal controls (Hu et al., 2004). The results of our study have demonstrated elevated levels of CRP in both the hyperglycaemic and normoglycaemic individuals which could be attributed to the increased obesity rather than the abnormal glucose metabolism.

Like any other cross-sectional study, this study was subjected to limitations. No causative could be determined but only associations and correlations between T-cell markers and the studied parameters. Future longitudinal follow-up studies should be done to address this gap. Furthermore, although individuals with seasonal diseases such as flue and allergies that may affect the immune activation state were excluded in the study, we did not account for other inflammatory conditions and/or infections such as HIV and tuberculosis. Participants were only screened for clinical parameters and no viral or bacterial assays were performed. Additionally, the sample size on the effects of glucose on T-cell antigen expression was too small; a bigger sample size with subgroups (pre-diabetics, newly diagnosed T2DM and known T2DM) could have painted a more robust picture on the effect of glucose on T-cells antigen expression. On the flow cytometry technical aspects of this study, our compensation matrix values were solely determined by compensation beads. The inclusion of cells could have corrected the spill-over better. There were also a few methodological concerns in the functional aspect of this study. Firstly, the mere exposure of T-cells to glucose did not produce any dramatic change in marker expression as expected. The use of additional T-cell stimulants such as anti-CD3/CD28 or mitogen phytohaemagglutinin (PHA) could have addressed the actual effect of glucose on T-cell function. This needs to be considered in future studies. Secondly, the 45 minutes incubation time may have been too short. Most recent T-cell functional experiments have used about 4 hours of stimulation to detect real changes in marker expression. The calculated sample size (311) was quite higher than the number of samples ran in this study (69). This was due to high cost of mAbs and intermittent availability of samples as participants were recruited as they came in the clinic. These were huge limitations as it attributed to the difference in sample sizes for the different marker and made it impossible to perform any statistical analysis for other markers such as CD38.

The positives of this study are that the measurement of T-cell antigen expression using flow cytometry offers a highly sensitive and rapid method for both qualitative and quantitative measurements of immune activation. Moreover, numerous pre-analytical and analytical methodological considerations were made to minimize *in vivo* artefacts. These included the choice of anticoagulant and the sample processing time. Furthermore, the use of a T-cell viability marker in the glucose incubation protocol ensured that the cells that were being measured were viable. The incubation time and staining techniques applied in this study did not introduce any *in vivo* artefacts.

CHAPTER SIX

CONCLUSION

The results of this study have demonstrated no significant difference in the percentage of T-cells expressing activation antigens (HLA-DR and CD38) between individuals with hyperglycaemia and those with normal glucose levels at baseline. Similar results were obtained after analysing the expression of CD95 and PD-1. Furthermore, the results showed no correlations between T-cell antigen expression and glucose metabolism. However, there was a significant correlation between thee ultra-sensitive C-reactive protein (CRP) and the expression of HLA-DR on $CD4^+$ T-cells and CD95 on all T-cell subsets. In the subgroup of participants whose T-cells were incubated with glucose, there was a significant difference in the mean florescence intensity of HLA-DR on $CD4^+$ T-cells between the normoglycaemic and hyperglycaemic group at baseline. In addition, incubation with high concentrations of glucose significantly decreased the percentage of $CD4^+$ T-cells expressing CD95 in both groups.

The $CD4^+$ T-cells play an important role in the immune response, and future studies should continue to investigate the role of $CD4^+$T-cell subsets such as T_{regs}, Th17, Th1 and Th2 in the evolution of T2DM and the development of chronic inflammation. Furthermore, future studies should include more participants that can be sub-grouped into pre-diabetics, newly diagnosed T2DM and known T2DM. These studies could have important implications in understanding the progression of the disease and the development of immune dysregulation and complications such as cardiovascular diseases which are often associated with T2DM.

To conclude, we hypothesize that high concentrations of glucose suppress the immune system by decreasing the expression of $CD4^+$ T-cell activation markers which may lead to immune exhaustion. Furthermore, the chronic inflammation observed in T2DM may indicate the development of complications such as cardiovascular diseases and therefore immune monitoring could play an important role in therapeutic decisions to prevent these.

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APPENDICES

Appendix 1: Quality Control (QC)

Daily QC protocol was run on the instrument prior to any sample acquisition. Flow check fluorophores of uniformly sized microbeads (Beckman Coulter, Miami, FL) were used to standardise the optical path and laminar flow. The microbeads had predefined levels of fluorescence intensity and the half peak coefficient of variation (HP-CV) of each flow channel (FL1-FL8) was then measured. For the QC to pass, the HP-CVs of the flow channels should have been within set values and small since the beads fluoresce the same intensity (Fig 1). Levey-Jennings graphs were then used to plot the values obtained daily and determine action to implement should parameters fall outside the expected ranges.

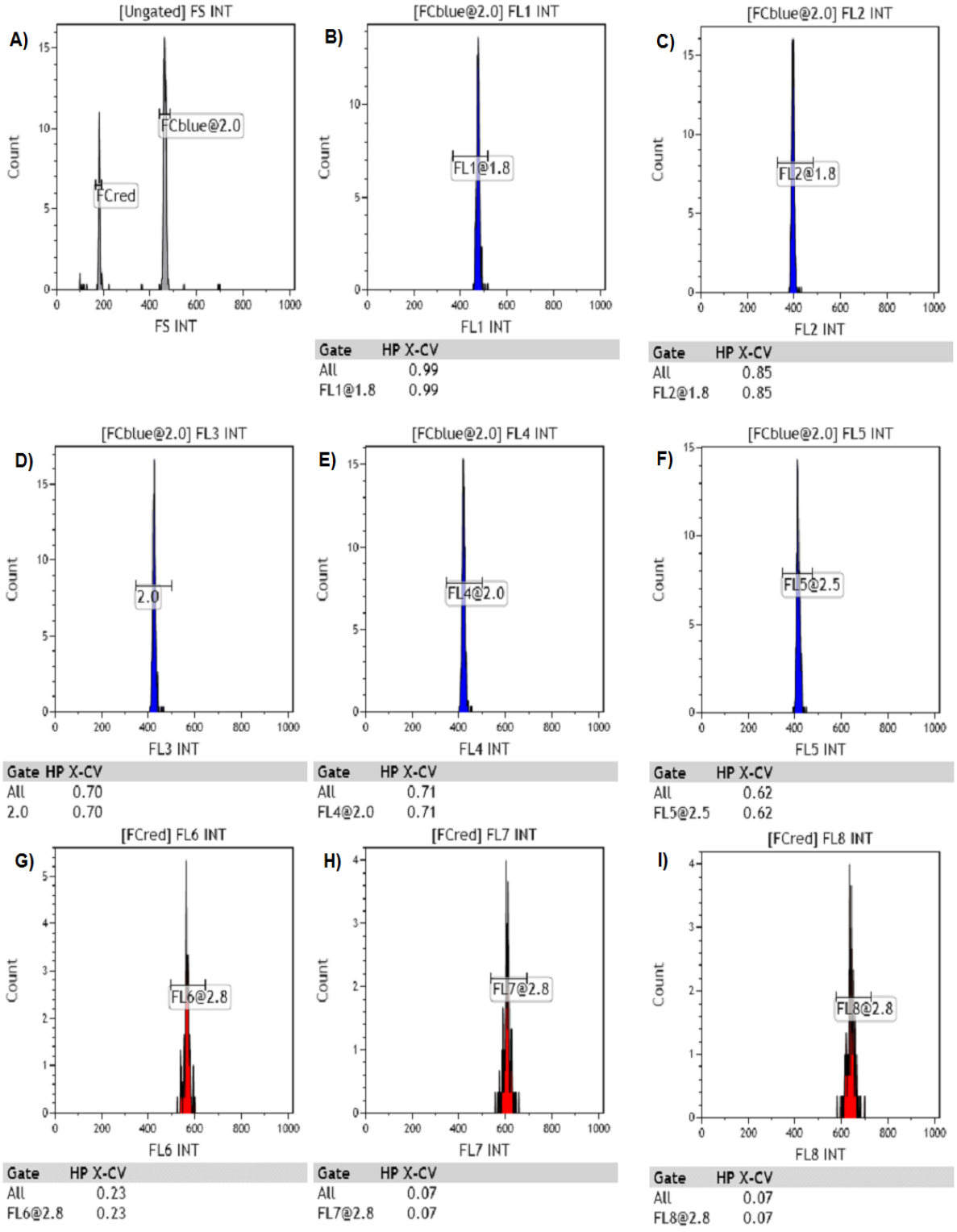

Figure 1: The figure illustrates plots of a QC that has passed. **Plot A** shows the primary gates on the red and blue fluorophores. **Plots B-F** illustrates flow fluorescent channels (FL1-FL5) on blue laser with HP X-CVs within the manufacturer's stipulated ranges. **Plot G-I** shows FL6-FL-8 channels on red lesser as well as their HP X-CVs within set ranges.

Appendix 2: Fluorescence Minus One (FMOs)

For markers that can be either up-regulated or down-regulated, such as HLA-DR, CD38 and PD-1 amongst other surface markers used in this study, FMO controls were used to distinguish between positive and negative events (Fig 1). An FMO control contains all the flourochromes in a panel except for the one that is being measured. Thus FMOs take into account the intrinsic autofluorescence of the cell and fluorescence due to the antibody binding detected in that flow channel (Tung et al., 2007).

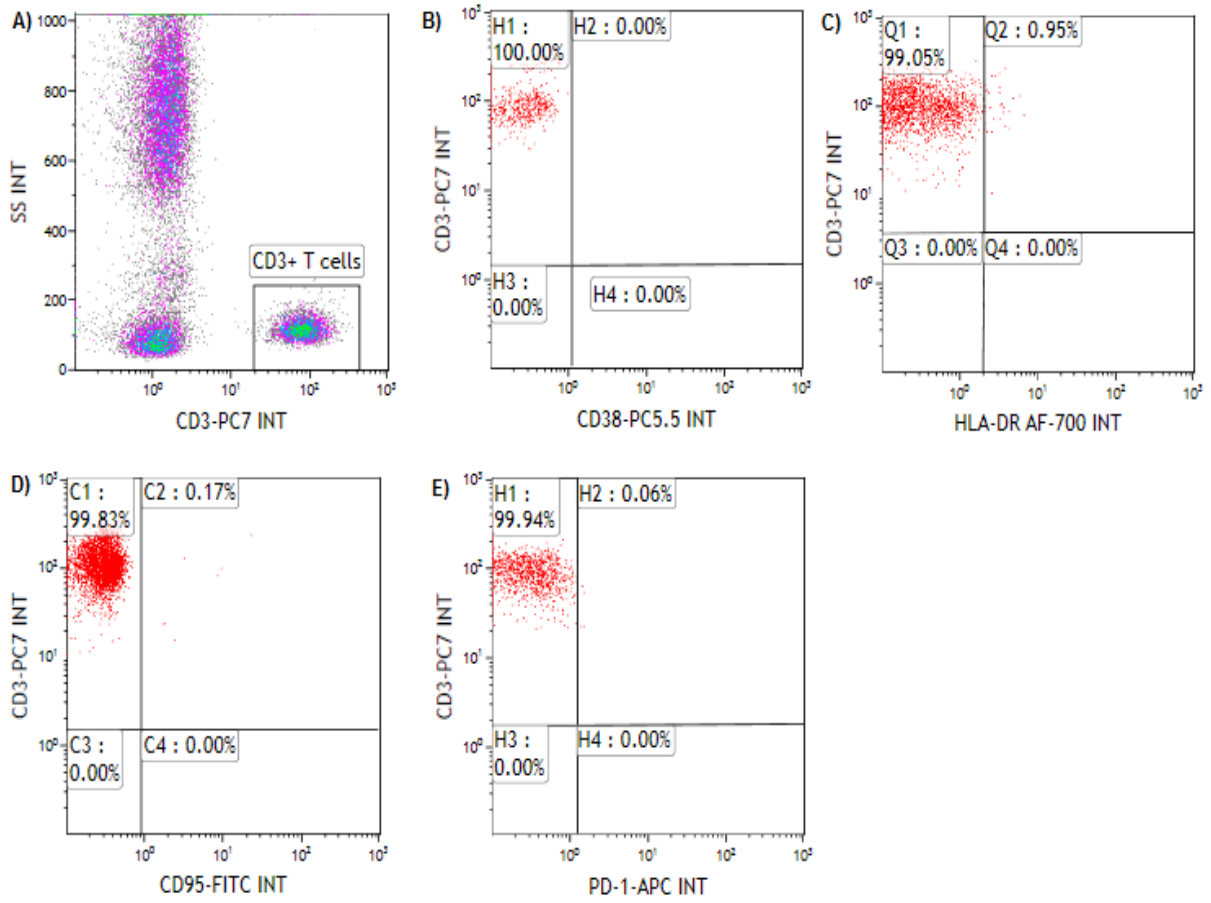

Figure 1: Florescence Minus One (FMO) controls. Plot A illustrates the primary gating for T-cells used in the FMOs. Plots B, C, D and E illustrate FMOs of CD38-PerCP-Cy5.5, HLA-DR-Alexa Fluor 700, CD95-FITC and PD-1-APC respectively, which were used to independently determine the boundaries between positive and negative events. A positive event was defined as greater than 1% and conversely, events less than 1% were considered to be negative

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Appendix 3: Titrations

Antibody titrations assays were done on all monoclonal antibodies (mAbs) used in this study to minimise non-specific antibody binding (Hulspas, 2010). Five (5) tubes per mAb titration were prepared using different antibody volumes from 0.5-2.5µl. The samples were then stained, incubated, lysed and acquired in the same manner as in the baseline protocols. Signal to noise ratios were then calculated using the formula below and the volume with the highest signal to noise ratio was chosen as the optimum antibody volume titration for each specific mAb used in a specific panel (Fig 1).

Formula: Signal to noise ratio = $\frac{Positive\ MFI}{Negative\ MFI}$ *x 100.*

This process was implemented on all the mAbs that were used in this study. Figure 1 below illustrates how the optimum volume of CD3 was determined.

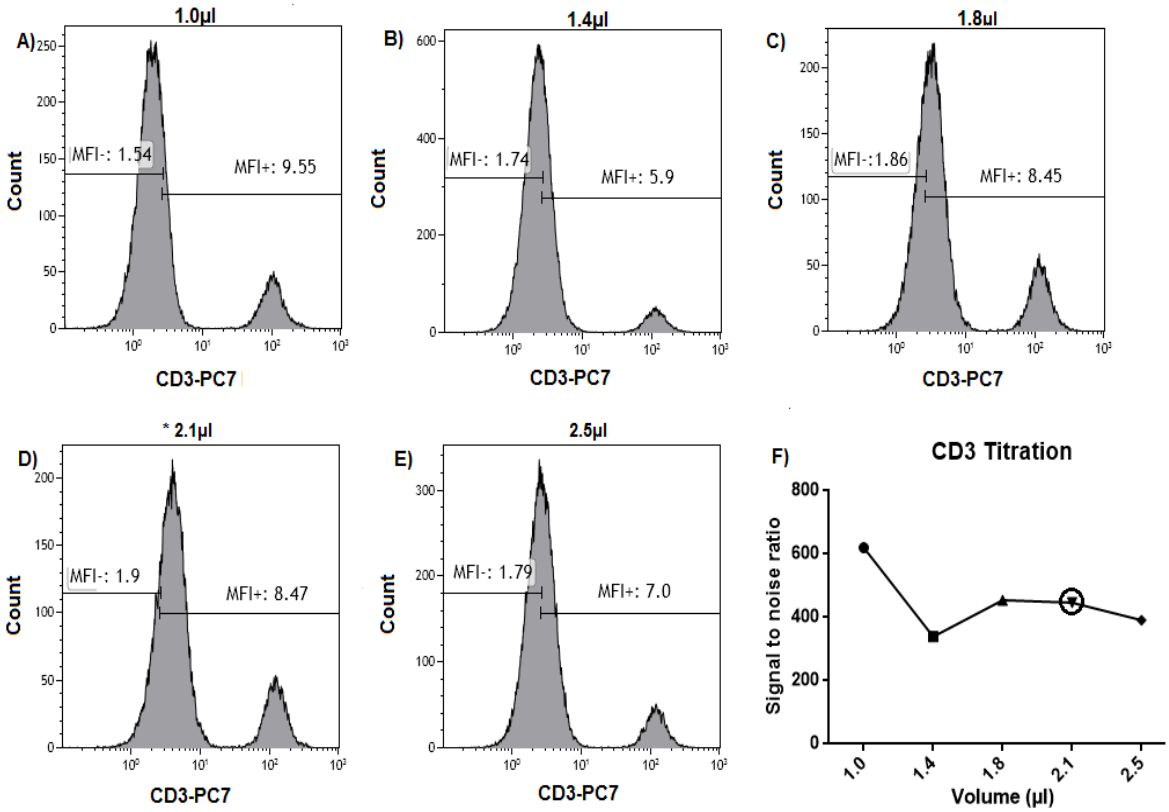


Figure 1: CD3-PC7 antibody titration. Plots **A-E** illustrates the positive and negative mean fluorescence index (MFI) of CD3-PC7 at different antibody volumes (1µl; 1.4µl; 1.8µl; 2.1µl and 2.5µl). The MFI's were then used to calculate the signal to noise ratio using the formula above and the results were plotted in graph **(F).** For this particular antibody (CD3-PC7), a volume of 2.1µl had the highest signal to noise ratio of 446% hence it was selected as the optimum volume of CD3-PC7 in the panels used in this study. This process was done on all other mAbs used in this study.

Appendix 4: Incubation Time Optimisation

Fifty microliters (50µl) of sodium citrate blood from three samples were added to 20µl of three different glucose concentrations (2mmol/l, 5mmol/l and 30mmol/l) mimicking hypoglycaemia, normoglycaemia and hyperglycaemia respectively. The samples were then incubated at four 15 minutes time intervals (0, 15, 30, 45 and 60 minutes). After each time interval, the samples were then stained by mAb cocktail, lysed and acquired in the same manner as explained in the staining protocol. T-cell recovery using percentage expression and mean fluorescence intensity (MFI) of $CD4^+$ T-cells was then calculated (Fig 1).

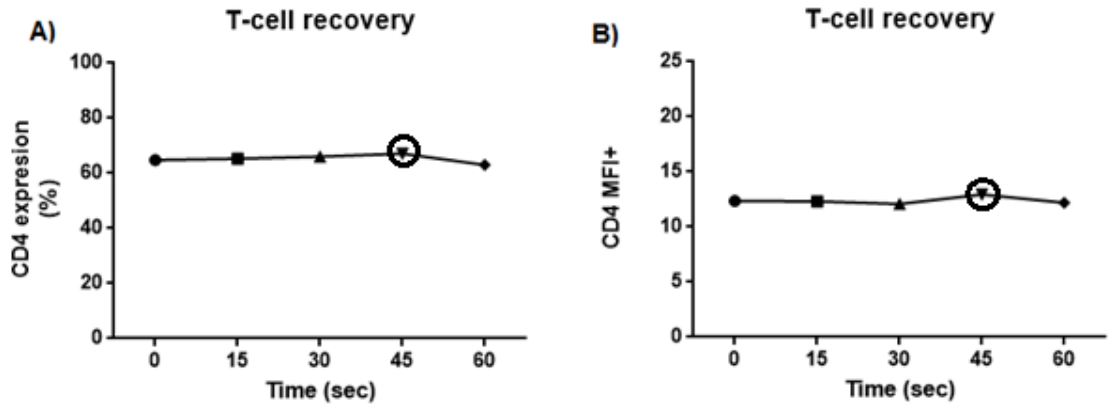


Figure 1: T-cell recovery at different incubation time intervals. **A)** Illustrates average $CD4^+$ expression of three (3) different samples at 15 minutes time intervals. **B)** The graph shows the average $CD4^+$ positive MFI from 0 to 60 minutes incubation intervals. T-cell recovery was optimum at 45 minutes based on $CD4^+$ expression and MFI as illustrated by both graphs above, hence 45 minutes was chosen as the incubation time for the functional test protocol.

Appendix 5: Viability Marker Control

The T-cell viability control test was done to investigate whether or not, the 45 minutes incubation time had any influence on cell survival. For the test control, 100µl of citrate blood were placed in a water bath at 65^{o}C for 5 minutes. 50µl of the heat-shocked cells were then stained and acquired in parallel with the unstained fresh cells (Fig 1A). An unstained sample was used to discriminate positive events from negative (Fig 1B). Three (3) samples were then incubated at 37^{o}C for 45minutes in the 3 different glucose concentrations (2mmol/l, 5mmoml/l and 30mmol/l), stained using the stimulation protocol for both the activation and exhaustion panels with addition of Zombie NIR-APC/Cy7 fixable viability dye. This T-cell viability test demonstrated that the incubation time used in this study did not influence cell survival and viability (Fig 2).

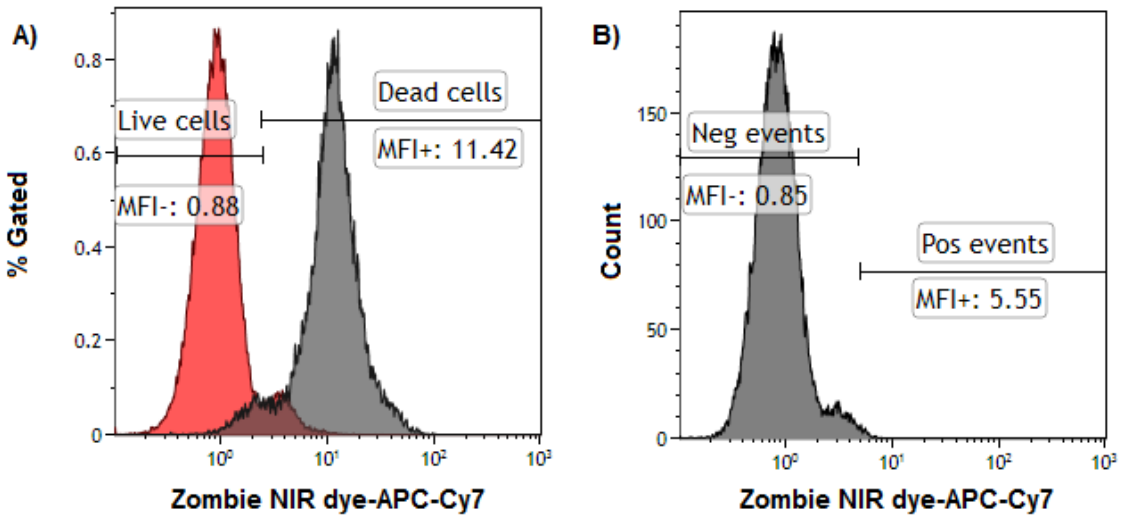


Figure 1: Viability marker control test. **Pot A)** Distinguishes live cells from dead cells using the Zombie NIR-APC/Cy7 dye. **Plot B**) Demonstrates how the negative events were distinguished from positive events using fresh unstained blood sample by the use of mean fluorescence intensity (MFI).

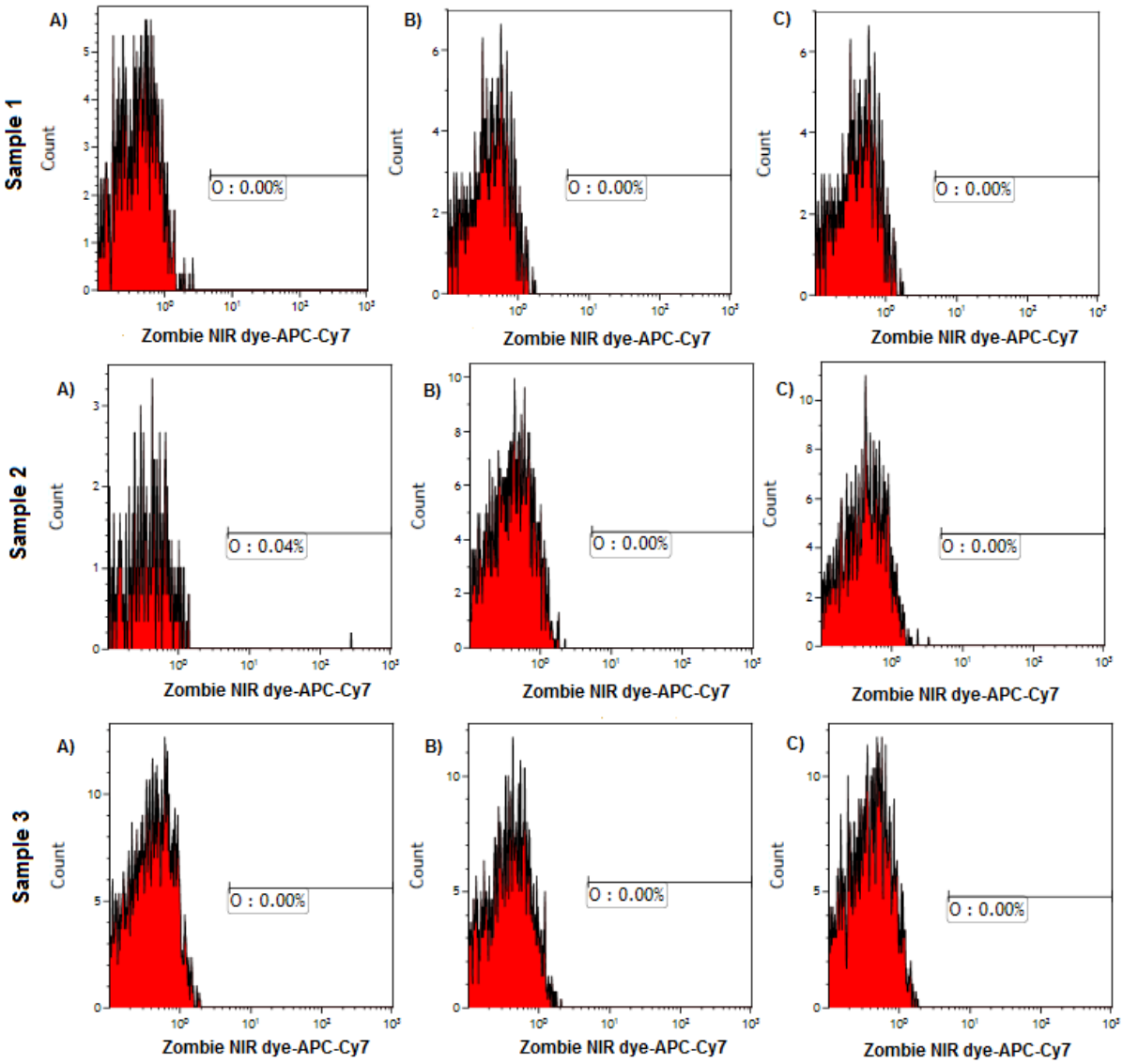
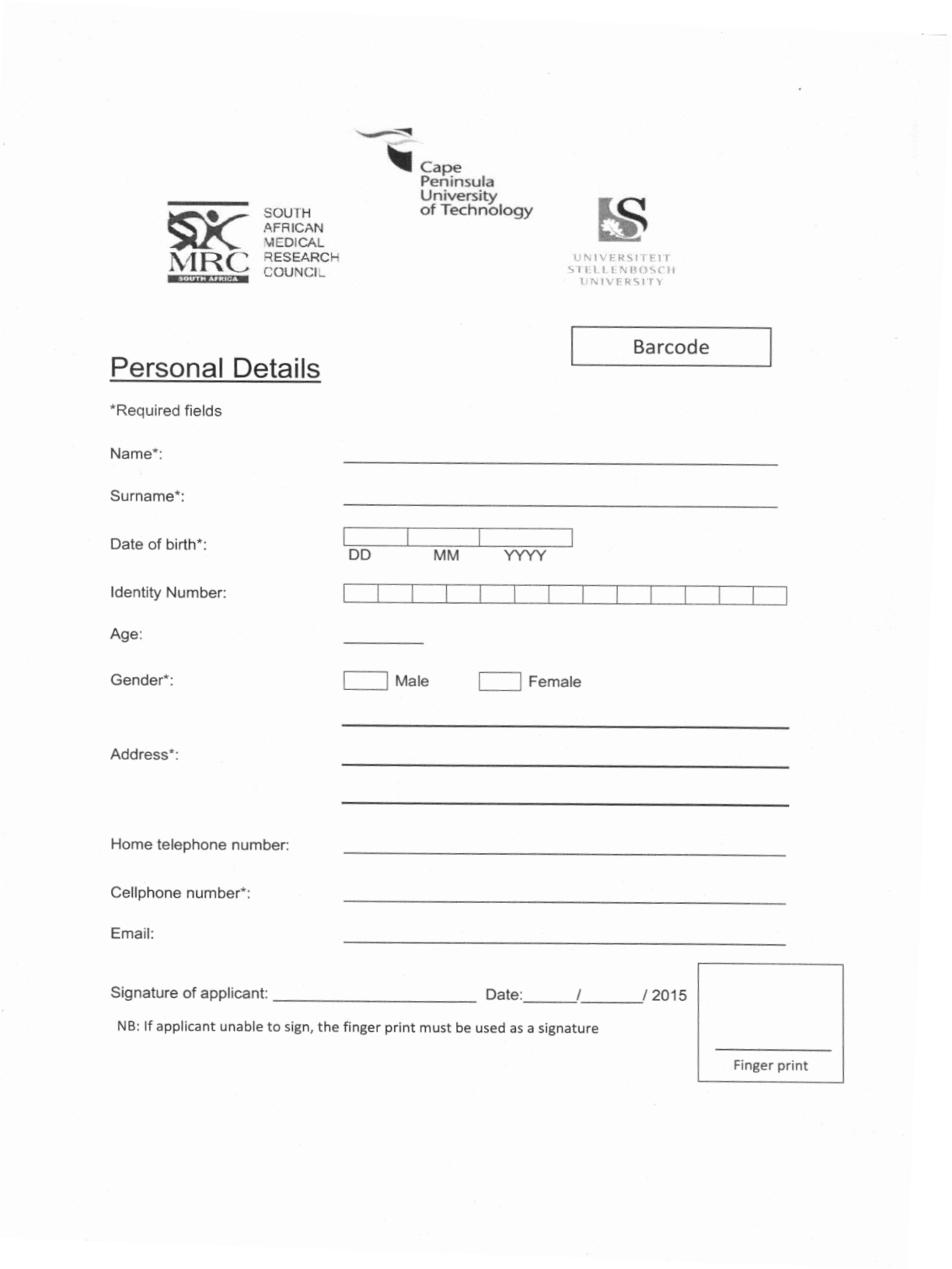


Figure 2: T-cell viability test. Percentages of dead cells using the Zombie NIR viability dye was measured in three (3) samples post 45 minutes incubation at 37^{o}C in 2mmol/l, 5mmol/l and 30mmol/l glucose concentration, represented by A, B and C respectively. The plots illustrate that the incubation time, the glucose concentrations and research methods applied in this study did not cause cells to die.

ADDENDA

ADDENDUM 1: Participant Information Leaflet and Consent Form



SOUTH AFRICAN MEDICAL RESEARCH COUNCIL

Cape Peninsula University of Technology

UNIVERSITEIT STELLENBOSCH UNIVERSITY

Barcode

Personal Details

*Required fields

Name*: _____

Surname*: _____

Date of birth*: ☐☐ ☐☐ ☐☐☐☐ (DD MM YYYY)

Identity Number: ☐☐☐☐☐☐☐☐☐☐☐☐☐

Age: _____

Gender*: ☐ Male ☐ Female

Address*: _____

_____

_____

Home telephone number: _____

Cellphone number*: _____

Email: _____

Signature of applicant: _____ Date:_____/_____/ 2015

NB: If applicant unable to sign, the finger print must be used as a signature

_____

Finger print

PARTICIPANT INFORMATION AND INFORMED CONSENT FORM FOR RESEARCH INVOLVING GENETIC STUDIES

TITLE OF RESEARCH PROJECT: PROGRESSIVE RESEARCH ON RISK FACTORS OF TYPE 2 DIABETES AND CARDIOVASCULAR DISEASES IN SOUTH AFRICA

REFERENCE NUMBER:

PRINCIPAL INVESTIGATORS:	**Professor Tandi Matsha (Cape Peninsula University of Technology)** **Professor Rajiv Erasmus (Stellenbosch University)** **Professor Andre Kengne (SA Medical Research Council)**
Co-Principle Investigator:	**Mr Mogamat Shafiek Hassan (Cape Peninsula University of Technology)**
Sub investigator:	**Dr Megan A Rensburg (Stellenbosch University)** **Dr Jan Esser (Stellenbosch University)**
Project manager:	**Dr Gloudina Maria Hon (Cape Peninsula University of Technology)**
ADDRESS:	**Obesity and chronic diseases of lifestyle** **Department of Biomedical Sciences** **Faculty of Health & Wellness Sciences** **Cape Peninsula University of Technology, Bellville**
CONTACT NUMBER:	**Prof T Matsha 021 959 6366 or email: matshat@cput.ac.za**
Ethics approval:	**Cape Peninsula University of Technology Ethics Reference number: CPUT/SW-REC 2015/H01** **University of Stellenbosch Ethics Reference number: N14/01/003**

We would like to invite you to participate in a research study that involves genetic analysis and possible long-term storage of blood or tissue specimens. Please take some time to read the information presented here which will explain the details of this project. Please ask the study staff or doctor any questions about any part of this project that you do not fully understand. It is very important that you are fully satisfied that you clearly understand what this research entails and how you could be involved. Also, your participation is ***entirely voluntary*** and you are free to decline to participate. If you say no, this will not affect you negatively in any way whatsoever. ***You are also free to withdraw from the study at any point, even if you do agree to take part initially.***

This research study has been approved by the ethics **Faculty of Health & Wellness Sciences of the Cape Peninsula University of Technology** and it will be conducted according to international and locally accepted ethical guidelines for research, namely the Declaration of Helsinki, and the SA Department of Health's 2004 Guidelines: *Ethics in Health Research: Principles, Structures and Processes.*

1. What is Genetic research?

Genetic material, also called DNA or RNA, is usually obtained from a small blood sample. Occasionally genetic material is obtained from other sources such as saliva or biopsy specimens. (A biopsy is a tiny piece of tissue that is cut out e.g. from the skin or from a lump, to help your doctor make a diagnosis.) Genes are found in every cell in the human body. Our genes determine what we look like and sometimes what kind of diseases we may be susceptible to. Worldwide, researchers in the field of genetics are continuously discovering new information that may be of great benefit to future generations and also that may benefit people today, who suffer from particular diseases or conditions.

2. What does this particular research study involve?

This research study seeks to address the increasing problem of diabetes and cardiovascular diseases such as heart attack and stroke amongst the mixed ancestry or coloured population of South Africa. In this study we shall identify people with diabetes and those at high risk of diabetes as well as investigate the environmental and genetic risk factors that predispose some individuals to the development of diabetes and cardiovascular diseases. Examples of environmental factors include body weight, diet, and physical activity. Additionally, this project aims to investigate whether oral health is a risk factor for diabetes and cardiovascular diseases. In this study we shall investigate whether some individuals have early cardiovascular diseases by using an ultrasound machine. This project also aims to collect genetic material (blood) to analyze for certain variants and to store excess material for future research. When a large group of patients with similar diseases has been collected, meaningful research into the disease processes may become possible.

3. Why have you been invited to participate?

Our research team has previously conducted a similar research study involving the coloured community and found out that more that 18 out of 100 individuals had diabetes but did not know. We also found that some of the risk factors associated with diabetes in other populations were not necessary the same as those affecting the coloured population of South Africa. You have therefore been invited to take part in this research study to assist in establishing the risk factors for diabetes and cardiovascular diseases affecting the coloured people of South Africa.

4. What procedures will be involved in this research?

A. You will be requested to provide information about your medical history, family history and information on eating, drinking and smoking habits. Completion of the questionnaire will take no longer than 30 minutes.
B. You shall be requested to provide a record of the medication you are currently taking, therefore if you are taking chronic medication, you shall be requested to provide this to the research team to record the medication.
C. Measurement such as weight, height, waist and hip will be done.
D. Fasting Venous Blood (20ml) will be collected thereafter you will be asked to drink a glucose solution (glucose content 75g). After two hours another venous blood (10ml) will be collected. The blood will be used to determine whether you have diabetes or you are at high risk for developing diabetes.
E. The other tests that will be determined from your blood sample are: Cholesterol, triglycerides, creatine levels to assess your kidney function, liver enzymes to assess your liver, and biochemical markers for inflammation.
F. A finger prick blood sample (a drop of blood), to be taken at the same time of the first venous blood sample, may also be required from you. The finger prick blood sample will be used to test for diabetes or the risk of developing diabetes on a point-of-care test instrument. Researchers will compare the

finger prick point-of-care diabetes test with that of the send away venous blood laboratory test and would be able to establish whether the point-of-care test provides the same accurate results as that of the laboratory. Point-of-care testing may in the future be used to provide fast and accurate results without the need to send blood away to a laboratory for processing. This may be of benefit to people undergoing testing for diabetes as results would be available within a few minutes.

G. The remainder of the blood sample will be used for genetic and future research studies. The serum and DNA may be stored for several years until the technology for meaningful analysis becomes available. No pharmaceutical agent (medication) will be tested in the study.

H. For oral health, research study personnel will extract wooden toothpick, flocked brush, and mouthwash saliva samples from you to test for the presence of Porphyromonas gingivalis as an indicator for periodontal disease. Flocked brush and wood toothpick sampling will involve inserting devices in the subgingival crevice between the last upper premolar and the first upper molar. The device will sweep down the anterior surface of the first upper molar with the direction of motion away from the gum to minimize any potential discomfort. Mouthwash sampling will involve rinsing with 10 ml sterile saline solution for 20 seconds.

I. Early cardiovascular diseases will be performed by means of an ultrasound machine.

J. The research team will follow up on you on a yearly basis and some of these test may be repeated. The investigators wish to follow you up for your entire life. In the unfortunate event that you are deceased during the study period. The study team will review stats SA data and/or medical records to ascertain whether the cause of death was due to diabetes or cardiovascular diseases. ***. If you do not wish to be followed up on a yearly basis and your Statistics SA and/or medical records not to be accessed in the unfortunate event that you are deceased whilst being a participant in the study, you will have an opportunity to request that it be not accessed when you sign the consent form.***

K. Radio imaging techniques will be done on consenting subjects. These include **(i)** ultra sound to assess whether you have signs of early cardiovascular diseases, **(ii)** computed tomography scan (CT-scan) to accurately assess the fat content that is dangerous for cardiovascular diseases **(iii)** Dual-energy X-ray absorptiometry (DXA) devices will be used to study the morphology of the liver. These radio imaging techniques involve radiation which can be harmful if one is exposed excessively. For this study a low dose radiation will be used for acquisition of the images thereby minimizing radiation exposure to the participant. ***. If you do not wish to undergo any of these radio imaging techniques, you will have an opportunity to decline when you sign the consent form.***

5. Are there any risks involved in genetic research?

A slight bruising might occur after blood has been drawn from the arm but this will heal quickly. After the administration of the glucose solution, you may feel nauseous and dizzy in which case you must notify the medical personnel. A medical nurse or doctor will be present on all occasions. You may also learn that you have diabetes, in which case you will be referred to your health care giver with the results for further treatment and management. If during the study it is discovered that you have changes in your genes that may lead to a serious disease, a genetic counsellor at the expense of the principal investigators will counsel you. Radio imaging techniques such as the CT-scan involves radiation which can be harmful if one is exposed excessively. For this study a low dose radiation will be used for acquisition of the images thereby minimizing radiation exposure to the participant.

6. Are there any benefits to your taking part in this study and will you get told your results?

Your personal results will be made known to you ***only if they indicate*** that you may:

- Have diabetes, thereafter, you will be referred to your local health centre or general practitioner for further investigations and treatment.
- Have a condition or predisposition to developing diabetes that is treatable or avoidable e.g. by a lifestyle modification.

- Need genetic counselling.

➢ **However, participants with normal results who wish to know their results are free to contact the research team and their results will be given upon written request.**

7. How long will your blood be stored and where will it be stored?

The blood samples may be stored ***indefinitely*** to accommodate new technologies that may develop. In the event that a technology is not available in South Africa to analyse your blood sample, your blood specimen may be sent to another country with the technology either now or at a later date. However, if your specimen is to be sent to another country, permission to do so will be sought from relevant bodies. Your blood specimen will be stored at the Cape Peninsula University of Technology.

8. If your blood is to be stored is there a chance that it will be used for other research?

Your blood will only be used for genetic research that is directly related to Diabetes and cardiovascular diseases. Also if the researchers wish to use your stored blood for ***additional research in this field*** they will be required to apply for permission to do so from the ethics ***Faculty of Health & Wellness Sciences of the Cape Peninsula University of Technology. If you do not wish your blood specimen to be stored after this research study is completed you will have an opportunity to request that it be discarded when you sign the consent form.***

9. **How will your confidentiality be protected?**

Your identity will be recorded once and kept confidential throughout. This is to allow the principal investigators to convey information that may be beneficial to you. Access will be limited to the principal investigators by assigning a special study code to all your data and blood samples. This means that your sample will be identified with a special study code that will remain linked to your name and contact details. However, during the entire research study, your blood specimens will be anonymised and the research staff won't be able to associate it with your name and contact details. You shall also be supplied this code so that if at anytime the investigators need to contact you, you may only identify yourself using your special code. Any scientific publications, lectures or reports resulting from the study will not identify you.

Some insurance companies may mistakenly assume that taking part in research indicates a higher risk for disease. Thus no information about you or your family will be shared with such companies.

10. Will you or the researchers benefit financially from this research?

You will not be paid to take part in this study ***although your out-of-pocket expenses may be reimbursed.*** The expenses that will be covered by the research team are those that include transportation to a hospital radiography department should you consent to radio imaging.

Important information: In the unlikely event that this research leads to the development of a commercial application or patent, you or your family will not receive any profits or royalties, but profits will be reinvested into supporting the cause of further research which may bring benefits to you or your family and to the community, such as health screening, medical treatment, educational promotions, etc.

11. Is there anything else you should know or do?

You should inform your family practitioner or usual doctor that you are taking part in a research study.
You can contact

Prof T Matsha at 021 959 6366 or matshat@cput.ac.za,
If you have any further queries or encounter any problems, you can also contact the Cape Peninsula University of Technology Health and Wellness Sciences Research Ethics Committee,
Chairperson: Prof Engel-hills at 0219596570 or EngelhillsP@cput.ac.za or

You will receive a copy of this information and consent form for your own records if it is requested.

12. Declaration by participant

By signing below, I ..... agree to take part in a research project that includes genetic research study entitled (**PROGRESSIVE RESEARCH ON RISK FACTORS OF TYPE 2 DIABETES AND CARDIOVASCULAR DISEASES IN SOUTH AFRICA**).

I declare that:

- I have read or had read to me this information and consent form and it is written in a language with which I am fluent and comfortable.
- I have had a chance to ask questions and all my questions have been adequately answered.
- I understand that taking part in this study is voluntary and I have not been pressurised to take part.
- I have received a signed duplicate copy of this consent form for my records.

13. Tick the option you choose:

☐ I agree that my blood or tissue sample can be stored ***indefinitely*** after the project is completed but that it is anonymised with all possible links to my identity removed, and that the researchers may then use it for additional research in this or a related field. Once my sample is anonymised, my rights to that sample are waivered. My sample may be shipped to another laboratory in SA or abroad to be used in other research projects in this or a related field

OR

☐ I agree that my blood or tissue sample can be stored ***indefinitely,*** but I can choose to request at any time that my stored sample be destroyed. My sample will be identified with a special study code that will remain linked to my name and contact details. I have the right to receive confirmation that my request has been carried out.

OR

☐ Please destroy my blood sample as soon as the current research project has been completed.

14. Tick the option you choose:

☐ I ***consent*** that the research team may follow me up for yearly check-up ***AND*** in the unfortunate event that I am deceased whilst still part of the study, I ***consent*** that the team may access Statistics SA and/or my medical records to ascertain whether the cause of my death was due to diabetes or cardiovascular diseases.

OR

☐ I ***do not consent*** to follow me up for yearly check-up ***BUT*** in the unfortunate event that I am deceased whilst still part of the study, I ***consent*** that the team may access Statistics SA and/or my medical records to ascertain whether the cause of my death was due to diabetes or cardiovascular diseases.

OR

☐ I **do not consent** to follow me up for yearly check-up ***AND*** in the unfortunate event that I am deceased whilst still part of the study, I ***do not consent*** that the team accessing Statistics SA and/or my medical records to ascertain whether the cause of my death was due to diabetes or cardiovascular diseases.

15. Tick the option you choose: Radio Imaging

☐ I ***consent*** to ultra sound techniques to assess if I have early cardiovascular diseases

☐ I ***do not consent*** to ultra sound techniques that assess if I have early cardiovascular diseases

AND

☐ I ***consent*** computed tomography scan (CT-scan) to accurately assess the fat content that is dangerous for cardiovascular diseases

☐ I ***do not consent*** to computed tomography scan (CT-scan) that accurately assess the fat content that is dangerous for cardiovascular diseases

AND

☐ I ***consent*** to Dual-energy X-ray absorptiometry (DXA) used to study body composition.

☐ I ***do not consent*** Dual-energy X-ray absorptiometry (DXA) used to study body composition

Signed at (*place*) ..... on (*date*) .....

Finger print

....

Signature of participant **Signature of witness**

16. Declaration by investigator

I *(name)* ..... declare that:

- I explained the information in this document to .....
- I encouraged him/her to ask questions and took adequate time to answer them.
- I am satisfied that he/she adequately understands all aspects of the research as discussed above.
- I did/did not use a interpreter. (*If a interpreter is used then the interpreter must sign the declaration below.*

Signed at (*place*) ..... on (*date*) ..... 2015.

....

Signature of investigator **Signature of witness**

17. Declaration By Interpreter

I *(name)* ..... declare that:

- I assisted the investigator (*name*) ..... to explain the information in this document to (*name of participant*) ..... Using the language medium of Afrikaans/Xhosa.
- We encouraged him/her to ask questions and took adequate time to answer them.
- I conveyed a factually correct version of what was related to me.
- I am satisfied that the participant fully understands the content of this informed consent document and has had all his/her question satisfactorily answered.

Signed at (*place*) ..... on (*date*) ..... 2015.

....

Signature of interpreter **Signature of witness**

ADDENDUM 2: Ethical Clearance

Cape Peninsula
University of Technology

HEALTH AND WELLNESS SCIENCES RESEARCH ETHICS COMMITTEE (HW-REC)
Registration Number NHREC: REC- 230408-014

P.O. Box 1906 • Bellville 7535 South Africa
Symphony Road Bellville 7535
Tel: +27 21 959 6917
Email: sethn@cput.ac.za

17 September 2015
REC Approval Reference No:
CPUT/HW-REC 2015/H26

Faculty of Health and Wellness Sciences – Biomedical Sciences Department

Dear Mr Tawanda Maurice Nyambuya

Re: APPLICATION TO THE HW-REC FOR ETHICS CLEARANCE

Approval was granted by the Health and Wellness Sciences-REC on 17 September 2015 to Mr Nyambuya for ethical clearance. This approval is for research activities related to staff research in the Department of Biomedical Sciences at this Institution.

TITLE: Chronic immune activation markers in mixed ancestry subjects with hyperglycaemia from Western Cape, South Africa.

Supervisor: Dr Glenda Davison
Co-Supervisor: Dr Bongani Nkambule

Comment:
Approval will not extend beyond 18 September 2016. An extension should be applied for 6 weeks before this expiry date should data collection and use/analysis of data, information and/or samples for this study continue beyond this date.

The investigator(s) should understand the ethical conditions under which they are authorized to carry out this study and they should be compliant to these conditions. It is required that the investigator(s) complete an **annual progress report** that should be submitted to the HWS-REC in December of that particular year, for the HWS-REC to be kept informed of the progress and of any problems you may have encountered.

Kind Regards

Mr. Navindhra Naidoo
Chairperson – Research Ethics Committee
Faculty of Health and Wellness Sciences

Addendum 3: Research Outputs

Conference Presentations

Poster presentations

S.T.E.M congress, October 27th- 29th 2015, Johannesburg, South Africa

Fas signalling pathway in hyperglycaemia. Nyambuya T.M[1], Nkambule BB[1], Davison GM[1], Matsha TE[1]. Department of Biomedical sciences, Faculty of Health and wellness sciences, Cape Peninsula University of Technology, Bellville, South Africa[1].

Oral presentations

Laboratory Medicine congress, May 18th – 21st 2017, Durban, South Africa

PD-1 and HLA-DR expression in Hyperglycaemia. Nyambuya, TM[1], Davison, GM[1], Nkambule, BB[1, 2], Matsha T[1]. Department of Biomedical sciences, Faculty of Health and wellness sciences, Cape Peninsula University of Technology, Bellville, South Africa[1]. School of Laboratory Medicine and Medical Sciences, University of KwaZulu-Natal, Durban, South Africa[2].