UNIVERSITY OF KWAZULU-NATAL ™

INYUVESI YAKWAZULU-NATALI

INVESTIGATING MARKERS OF T CELL ACTIVATION DUE TO CHRONIC INFLAMMATION IN TYPE 2 DIABETES

Submitted in partial fulfillment of the requirements for the degree of Master of Medical Science, Department of Human Physiology, School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu-Natal

By

NONKULULEKO MBATHA (BTech)

217065918

2020

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Supervisor: Dr. BONGANI B. NKAMBULE

February 2020

DECLARATION OF ORIGINAL WORK

I, Ms. Nonkululeko Avril Mbatha, declare as follows:

1. That the work in this thesis has not been submitted to UKZN or other tertiary institutions for purposes of obtaining an academic qualification, whether by myself or any other party.
2. That my contribution to the project was as follows: I was the primary author of the project proposal for this thesis, as well as the thesis. I collected data and analyzed it. After that, I drafted the manuscript included in the thesis. I am also the first author of the manuscript submitted to BMC Endocrine disorders.

3. That the contribution of others to the project was as follows: a. Dr. Bongani B. Nkambule was the supervisor and spearheaded the direction of the project. He was the link during the submission and acceptance of the project proposal to the Biomedical Research Ethics Committee, Westville Campus. He also contributed with the conception of the manuscript, added to the content, and approved the final versions submitted to the journal.

Candidate: NONKULULEKO Avril MBATHA,

Signed  Date:…… March 09, 2020…..

As the candidate's supervisor, I agree with the submission of the thesis:

Dr. BONGANI B. NKAMBULE

Signed  … Date ……11 March 2020……..

DEDICATION

I am dedicating this thesis to my beloved family, who have meant and continue to mean so much to me.

First and foremost, to my mother, whose love for me knew no bounds and who taught me the value of hard work. To my grandmother, who raised me, loved me, and taught me to always be objective in life.

I love you all beyond words.

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RESEARCH OUTPUT

MANUSCRIPT SUBMITTED FOR PUBLICATION

Authors contributed equally in drafting this manuscript

Evaluation of immune activation and programmed death ligand-1 expression on T cells following a short-term high-fat diet

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LIST OF ABBREVIATIONS

AICD: Activation-induced cell death

AGEs: Advanced glycation end products

APC: Antigen-presenting cell

BMI: Body Mass Index

CD: a cluster of differentiation

CRP: C-reactive protein

CTLA-4: Cytotoxic T-lymphocyte-associated protein 4

CVD: Cardiovascular disease

DM: Diabetes Mellitus

DoH: Department of Health

HLA-DR: Human Leucocyte Antigen-D related

HG: Hyperglycemia

IDDM: Insulin-dependent diabetes mellitus/ NIDDM: Non-insulin dependent diabetes mellitus

IL: Interleukin

INF: Interferon

IR: Insulin Resistance

MHC: Major Histocompatibility

NF-kB: Nuclear factor-kappa B (transcriptional factor)

PD-1: programmed cell death-1

T2D: Type 2 Diabetes

TCR: T cell receptor

TLR: Toll-like receptors

TNF: Tumor necrosis factor

Teff: Effector T-cells

Treg: Regulatory T-cells

WAT: White adipose tissue

WHO: World Health Organization

ABSTRACT

Background: Type 2 diabetes (T2D) is amongst the leading causes of mortality associated with non-communicable diseases. Insulin resistance, low-grade chronic inflammation, together with T cell activation, play an essential role in the pathogenesis of T2D and cardiovascular diseases. However, the role of T cells in the pathogenesis of T2D remains unclear.

Study Design and Methods: Thirty-four (n = 34) male C57BL/6 mice were obtained from the UKZN, Biomedical Resource Unit in Westville. The mice were randomized into a six-week low-fat diet (LFD) fed control group and a high-fat diet (HFD) fed experimental group. Body weights, plasma, glucose levels, T cell activation, and exhaustion markers were compared amongst the two groups before and post-treatment with either low dose aspirin (LDA), a combination of low-dose aspirin and metformin or Clopidogrel.

Results: HFD-fed mice demonstrated weight gain, elevated plasma glucose ($p = 0.008$), and insulin levels ($p=0.026$) within two weeks of consuming the diet. CD69 expression levels on T cells were lower in the HFD-fed group when compared to the LFD-fed group ($p = 0.0208$). All the treated groups demonstrated elevated levels of CD69 expression on T cells. Only the LDA treated group showed a tendency towards a reduction in PD-1 levels of expression on T cells when compared to the untreated HFD-fed group ($p = 0.0711$).

Discussion: Impaired glucose tolerance is associated with increased T cell activation in prediabetes. The HFD fed mice had reduced levels of CD69 expression on T cells, indicating T cell activation and chronic inflammation. CD69 modulates T cell egress to inflamed tissue. Expression levels of CD69 in T cells was ameliorated post-treatment with LDA, a combination of LDA and metformin or Clopidogrel, therefore preventing cardiovascular complications.

Conclusion: T cell-mediated inflammatory responses can be attenuate by treatment with LDA, a combination of LDA and metformin or Clopidogrel in T2D. Increased T cell activation markers in hyperglycemic conditions demonstrated that chronic activation of T cells poses a risk to develop T cell dysfunction and T2D. Therefore, more focus should be given to the chronic activation of T cells in order to prevent the development of T2D rather than only focusing on obesity as a potential predisposing factor.

Keywords: Type 2 diabetes, CD69, PD-1, T cell activation, Prediabetes, chronic inflammation.

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CHAPTER ONE: INTRODUCTION

This chapter outlines the general topic, gives some background and provides a review of the literature related to the topic (section 1.0). Section 1.1. Background of the study, Section 1.2. Outlines the Problem statement. Section 1.3. Describes the Aim of the stud, section 1.4. States objectives of the study, section 1.5. Includes an outline of the remaining chapters of the thesis or layout of the thesis.

CHAPTER ONE: INTRODUCTION

1.0. Introduction

Diabetes Mellitus (DM) is a group of metabolic disorders of various etiologies. Individuals with DM present with hyperglycemia due to defects in the secretion of insulin or the action of insulin (Chatterjee *et al.*, 2017). DM has become a global health problem, especially in developing countries, with an estimated 366 million people that are affected worldwide (American Diabetes Association, 2013). A secondary metabolic disorder that has rapidly become a considerable burden in South Africa due to unhealthy lifestyles and accounting for more than 90% of diabetic cases is Type 2 diabetes (T2D) (Pheiffer *et al.*, 2018). T2D is characterized by chronic hyperglycemia due to insulin resistance, which induces systemic low-grade inflammation and activation of the immune system (Donath and Shoelson, 2011; American Diabetes Association, 2013).

Obesity is a significant risk factor associated with insulin resistance, and it is also a predisposing factor of T2D. Previous studies reported increased levels of molecules that play a role in modulating chronic inflammation in adipose tissue by inhibiting insulin signaling such as fibrinogen, plasminogen activator inhibitor 1 (PAI-1), C-reactive proteins (CRP), serum amyloid A (SAA), proinflammatory cytokines such as tumor necrosis factor (TNF-α), interleukin (IL)-1β, Interferon-gamma (IFN-γ) and IL-6. (Bastard *et al.*, 2000; Bruun *et al.*, 2006). Insulin resistance caused by obesity is involved in the pathogenesis of T2D and CVDs because of the action of resistin and leptin, which are inflammatory mediators derived from adipocytes that promote inflammation (Sell, Habich and Eckel, 2012). A previous study reported increased levels of activated T cells in the adipose tissue of obese mice, while another study reported on obesity-induced expression of Major Histocompatibility (MHC) class II on adipocytes which activated $CD4^+$ T cells to stimulate adipose tissue inflammation (Deng *et al.*, 2013; Shirakawa *et al.*, 2016). These findings suggest that $CD4^+$ T plays a crucial role in obesity-induced inflammation. Some studies have reported that T2D related complications are associated with insulin resistance, chronic inflammation, and T cell activation (Guarner and Rubio-Ruiz, 2014; Nadeem *et al.*, 2015; Nyambuya *et al.*, 2018).

T cell dysfunction is present in prediabetes (Zeng *et al.*, 2012). It has been suggested to be due to persistent insulin resistance that maintains an activated state of T cells (O'Sullivan et al., 2016). However, the actual immune phenotype of activated T cells in the pathogenesis of T2D remains unaddressed. Therefore, understanding the exact role of activated T cells in the pathogenesis of T2D may be beneficial in developing novel therapeutic approaches to modulate metabolic inflammation and insulin resistance. This study aimed to define markers of T cell

activation and T cell exhaustion in a prediabetic model by assessing immune function and T cell dysfunction. The outcome of this project will provide evidence that can be used to identify markers that might assist the public health sector in facilitating early diagnosis and prevention of T2D, particularly in resource-limited areas.

1.1. Background of the study

There has been little work in Africa on T-cell-mediated T2D pathogenesis, which has become an important emerging area of interest (Fay, Larson and Jameson, 2016). An expanding body of knowledge refers to the inflammation-driven pathogenesis of T2D (Alberti and Zimmet, 1998). T2D-related deaths are one of the major causes of non-communicable disease mortality In Africa (Mathers and Loncar, 2006).

T2D is a secondary hyperglycemic abnormality linked to an increased risk of thrombosis and ca rdiovascular disease (CVD) (Pickup, 2004). A wealth of evidence points to T cell-mediated inflammation in the pathogenesis of T2D (Badawi et al., 2010; Defuria et al., 2013; Qiao et al., 2016; Moro-garcía et al., 2018). Despite all this evidence, there is limited information on T cell immune-phenotype in the pathogenesis of T2D. Hence, this study aimed to evaluate T cell activation and exhaustion markers in prediabetes.

1.2. Problem statement

Diabetes is a category of metabolic diseases that are identified by hyperglycemia, that contribute s to chronic T lymphocyte activation (Pickup, 2004; American Diabetes Association, 2013). Information on the specific immune mechanisms involved in the pathogenesis of T2D remains scarce. It is uncertain how the inflammatory mechanism is triggered and is facilitated by effector T cells.

A crucial role of the adaptive immune system has been established in influencing local and systemic inflammation together with insulin resistance in T2D; however, the underlying T cell involvement is not fully understood. In Africa, the public health sectors are over-populated by patients, challenged by healthcare staff shortages, and delays in treatment administration, which impacts negatively on the public. This study intended to provide additional information in promoting a clearer understanding of T cells' function in the T2D pathogenesis.

1.3. Aim of the study

To investigate T cell activation and exhaustion in a state of impaired glucose tolerance using a HFD-fed animal model of prediabetes

1.4. Objectives of the study

- To optimize a flow cytometry-based assay to determine T cell-mediated immune activation, T cell exhaustion in prediabetes.
- To determine the levels of T cell activation and exhaustion.
- To determine the function of $CD4^+$ and $CD8^+$ T cells in the pathogenesis of T2D using an animal model of prediabetes.

1.5. Layout of the thesis

This thesis is presented in a manuscript format, as per the UKZN requirements, with the Results and Discussion being described in the manuscript that has been prepared for publication. It is structured as follows:

Chapter 1. Introduction: *This chapter gives a Background of the study, problem statement, aim of the study, objectives of the study, and layout of the thesis.*

Chapter 2. Literature review: *this chapter outlines a brief overview of the pathogenesis of Type 2 diabetes, Hematopoiesis, the Immune response in Type 2 diabetes, the Inflammatory Response in type 2 diabetes, T cell activation in the pathogenesis of Type 2 diabetes and T cell regulation in type 2 diabetes.*

Chapter 3. Manuscript - Research article: *"Evaluation of immune activation and programmed death ligand-1 expression on T cells following a short-term high-fat diet". This chapter provides an original research manuscript on markers of T2D associated with chronic inflammation in prediabetes and the prevention of T2D. A manuscript was written and submitted to BMC Endocrine disorders.*

Chapter 4. Synthesis chapter: *This chapter provides general discussions, general conclusion of the study and recommendations for future studies.*

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CHAPTER TWO: LITERATURE REVIEW

This chapter outlines the general topic, gives some background and provides a review of the literature related to the topic (section 2.0). Section 2.1. Outlines the Pathogenesis of Type 2 diabetes. Section 2.2 describes the hematopoiesis, section 2.3 states the Immune response in Type 2 diabetes, section 2.4 outlines the Inflammatory Response in type 2 diabetes section 2.5 includes T cell activation in the pathogenesis of Type 2 diabetes and finally, section 2.6. Talks about T cell regulation in type 2 diabetes.

CHAPTER TWO: LITERATURE REVIEW

2.0. Introduction

Type 2 diabetes (T2D) has become an epidemic worldwide that accounts for increased morbidity and mortality. T2D exerts an enormous financial strain on the health care system (Cruz, Fernandes, and Gomes, 2012). Immune dysfunction and chronic adipose tissue inflammation were associated with the pathogenesis of T2D (Lontchi-Yimagou *et al.*, 2013).

Previous studies found T2D more predominant in adults with risk factors such as obesity, hypercholesterolemia, and hypertension (Kaneto, 2015; Ansari *et al.*, 2003). Obesity is associated with the disruption of glucose homeostasis leading to insulin resistance and the development of T2D (Kaneto, 2015; Ozougwu, 2013). Besides, an expansion of adipocytes results in the recruitment and infiltration of macrophages of adipose tissues. This process activates inflammation, thus causing obesity-induced insulin resistance (Carmeliet, 2005; Herder *et al.*, 2007; Zeyda and Stulnig, 2009). Elevated circulating levels of pro-inflammatory cytokines such as TNFα and IL-6 negatively affect the insulin signaling cascade' leading to 'obesity-induced insulin resistance' (Lauterbach and Wunderlich, 2017). The secretion of IL-17 pro-inflammatory cytokines activates CD4+ T cells to stimulate the activation of T cells by the NF-kB pathway (Isomaki *et al.*, 2005). Some studies have identified a link between obesity-induced insulin resistance, chronic T cell activation, and T2D (Lontchi-Yimagou *et al.*, 2013). Prolonged immune activation ultimately leads to T cell exhaustion and activation-induced cell death (AICD) (Barber *et al.*, 2006). Dysfunction of T cells results from prolonged exposure to proinflammatory cytokines such as TNF-α (Cope, 2002).

Overall, these studies show that T-cell activation may be triggered by hyperglycemia, which is induced by the secretion of pro-inflammatory cytokines. The mentioned process eventually leads to chronic inflammation and T2D, although evidence on the activated T-cell immune phenotype is still restricted. There is uncertainty about how the T-cell-mediated adaptive immune response plays a role in the pathogenesis of T2D (Lontchi-Yimagou *et al.*, 2013).

This work aimed at illustrating and identifying markers of T2D-associated with chronic inflammation in prediabetes to promote early treatment, intervention, and prevention T2D. The significance of T cell investigation studies in obesity enables the early identification of immune dysfunctions, thereby preventing metabolic disorders and associated complications (Cruz, Fernandes, and Gomes, 2012).

Restoring T cell homeostasis may be beneficial for therapeutic purposes (Ali *et al.*, 2001; Goronzy and Weyand, 2001; Cope, 2002). Therefore, this study will be able to provide

information that can be used in determining the link between chronic inflammation and insulin resistance, which leads to T2D. This study will also contribute to existing literature and knowledge by providing facts based on the pathogenesis of chronic inflammation through the assessment of immune markers of activated T lymphocytes as a risk factor for T2D.

2.1. Pathogenesis of Type 2 diabetes

T2D pathogenesis is associated with insulin resistance development, which is accompanied by hyperglycemia, chronic inflammation, and T-cell dysfunction (Cruz, Fernandes, and Gomes, 2012).

2.1.1. Other categories of diabetes mellitus

Most diabetic cases fall into type 1 diabetes (T1D) or T2D category with other categories that are not very common (Donald *et al.*, 2015). T1D accounts for only 5–10% of diabetes cases and is more prevalent in children and young adults (King, 2008). T1D is defined as an autoimmune disorder that results from the auto-destruction of β-cell pancreatic cells, resulting in a total loss of insulin secretion and insulin deficiency (Diabetes, 2009). Symptoms of T1D include fatigue, vision blurriness, polyuria, and weight loss (Itariu and Stulnig, 2014). Idiopathic diabetes is a highly inherited type of T1D. It does not have known etiologies; patients with permanent hypoinsulinemia have varying degrees of insulin deficiency between episodes and are vulnerable to ketoacidosis. Autoimmunity is not proven (Diabetes, 2009).

2.1.2. Type 2 diabetes (T2D)

T2D development requires a combination of genetic factors associated with impaired insulin resistance, insulin secretion (dysfunctional pancreatic β cells), and obesity-associated with plasma glucose homeostasis disruption (Kaneto, 2015; Itariu and Stulnig, 2014). In obesity, hypertrophy of adipocytes increases triglyceride storage, leading to apoptotic cell death and the secretion of adipokines that contribute to insulin resistance (Harford *et al.*, 2011; Donath and Shoelson, 2011; Defuria *et al.*, 2013). These are the cytokines released by macrophages (Lauterbach and Wunderlich, 2017). Resting T cells are noted to be devoid of insulin receptors. In the obesity-induced hyperglycemia, insulin activates T cells and maintains their responsiveness; therefore, defects in insulin action may lead to various metabolic disorders such as diabetes (Saxton et al., 2019).

Other studies have demonstrated that adipose tissue infiltration by macrophages, acute-phase proteins, coagulation factors, (such as plasminogen activator inhibitor 1 (PA-1) and fibrinogen 5), as well as interleukin-1 (IL-1) and TNF-α, is closely linked to chronic inflammation and

T2D related complications such as stroke and CVDs (Zeyda and Stulnig, 2009; Sultan *et al.*, 2009; Esser *et al.*, 2014; Chng *et al.*, 2015). Taken together, these studies suggested that insulin resistance, T cell activation, and chronic inflammation played a significant role in the development of T2D and increased risk of thrombotic events.

2.2. Hematopoiesis

Hematopoiesis is a process of blood cellular component formation by the hematopoietic stem cells (HSCs) in the bone marrow that takes place in two waves; the primitive wave which involves an erythroid progenitor that gives rise to erythrocytes and macrophages and the definitive waves which are multipotent and can give rise to all blood lineages from embryonic development and carry on throughout adulthood (Mikkola, 2006; Jagannathan-Bogdan and Zon, 2013).

2.2.1. T cell Hematopoiesis

Significantly, precursor T cells are a growing progenitor of lymphoid in the bone marrow, and they become circulating T cell precursors once they have entered the circulation and further migrate to the thymus to differentiate into naïve T cells together with early thymic precursors (ETPs) and early lymphoid precursors (Miyamoto *et al.*, 2002; Tu *et al.*, 2017). Most ETPs suppress their B-cell capacity and gain Notch activation for the development of T cells (Sambandam *et al.*, 2005; Alpdogan, Van Den Brink, and Brink, 2012).

Double negative (DN) thymocytes grow T-cell receptors (TCR) TCR-β and TCR-α loci, and then adequately express CD4 and CD8, resulting in double-positive (DP) expression of CD4+/CD8+. The expression of αβ TCR in the recombinant activator gene (RAG) regulates TCR αβ heterodimer surface expression until positive selection takes place (Han *et al.*, 2014). Hematopoiesis studies have contributed essential knowledge about the process of T cell development and have assisted in new therapies being developed for diseases and have become a great benefit to scientists and clinicians in better understanding processes underlying blood disorders and related complications (Miyamoto *et al.*, 2002; Tu *et al.*, 2017).

2.2.2. T cell maturation and tolerance

The maturation of stem cells of the bone marrow includes the development of immature cells, the expression of receptor chains by Variable Diversity, and Joining gene recombination and differentiation (Mikkola, 2006; Chaplin, 2010). T cells migrate to the thymus to become thymocytes for maturation which occurs in two processes, firstly, the positive selection of thymocytes which can recognize self-MHC and secondly, the adverse selection of thymocytes

where the ones that have TCRs of high avidity for class I and class II MHC molecules presenting self- antigens undergo programmed cell death or they become functionally inactive and are termed tolerant as shown in Figure 1. Thymocytes with low avidity for self-MHC peptide are selected as effector $CD4^+$ and $CD8^+$ T cells (Teff) that eventually migrate to secondary lymphoid organs to mature and enter the peripheral immune system (Delves and Roitt, 2000; Delves & Roitt, 2000; Tober *et al.*, 2018). Moreover, the immunological tolerance of self-reacting T-cells in the thymus and the peripheral lymphoid organs are termed central tolerance and peripheral tolerance, respectively (Romagnani, 2006).

Furthermore, the interaction of thymocytes with antigens favors the differentiation of regulatory T cells (Tregs), and they express molecules such as (CD5, CD25, CTLA-4, and Foxp3), which are associated with an activated state in Teffs (Povoleri *et al.*, 2013). This observation was also reported in a previous study that demonstrated elevated levels of Vβ6+ Tregs upon neonatal infection of mice with the murine mammary virus (Povoleri *et al.*, 2013). Upon exposure to an antigen, T lymphocytes are activated, proliferate, and differentiate into effector cells, which initiate an immune response (Olsen Saraiva Camara *et al.*, 2012). Moreover, based on these findings, it is only logical to conclude that the TCR-MHC affinity interaction of T cells facilitates their maturation, proliferation, survival, or apoptotic cell death (ACD) (Huseby *et al.*, 2005). The phenomenon of immunological tolerance is essential in certain aspects as its dysregulation may result in autoimmunity and immune dysfunction.

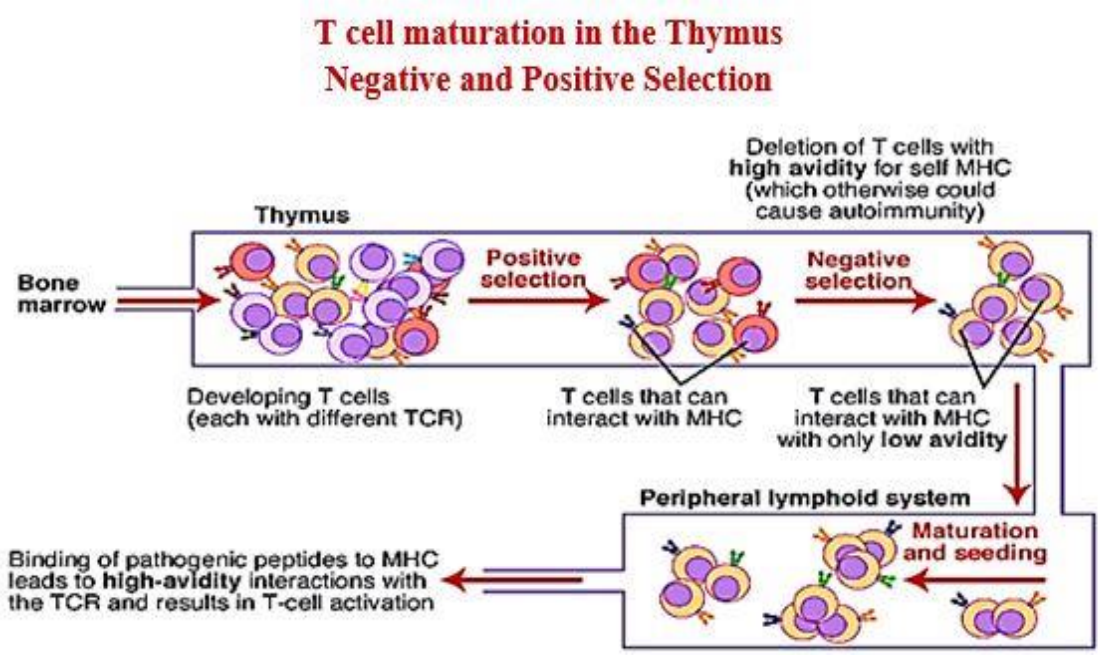

Figure 1. *T cell maturation in the thymus*

(Adapted from Cambridge University, 1999).

T lymphocytes are derived in the thymus, and their maturation occurs by positive and negative selection. T-cell receptors (TCRs) are generated from gene rearrangements. They are unique for each T cell, and those that cannot recognize self-major histocompatibility complex (MHC) molecules are allowed to die. Those that do are positively selected to mature in the thymus.

2.3. The Immune response in Type 2 diabetes

The immune system forms an integral part of T2D pathogenesis, which is classified within the innate and adaptive immune system. Previous studies have associated insulin resistance to increased levels of cytokines that are released in response to inflammation in the body (Ratajczak and Adamiak, 2015; Ratajczak et al., 2018).

2.3.1. The Innate Immune system

The innate immune system is described as the body's rapid first-line support, which is nonspecific and appears to lack immunological memory against threats to the environment such as microbial, physical, and chemical injuries (Parkin and Cohen, 2001; Ricardo, 2012). Furthermore, the innate immune system comprises phagocytic cells such as natural killer cells basophils, eosinophils, and mast cells that release inflammatory mediators upon activation (Figure 2) (Ratajczak and Adamiak, 2015; Ratajczak et al., 2018). The adaptive immune response is stimulated by the innate immune system upon binding of the exogenous molecules to toll-like receptor-4 (TLR-4) which triggers the release of pro-inflammatory cytokines, like

TNF-α, interleukin (IL)-1b and IL-6 (Kim *et al.*, 2015; Bilan *et al.*, 2009). These cytokines are primarily derived from activated macrophages. They can directly enhance insulin resistance in target tissues (Marc Y. Donath and Shoelson, 2011). The disruption of insulin homeostasis triggers the "inflammatory network" which is acute-phase inflammatory proteins such as CRP; serum amyloid-A (SAA); and pro-inflammatory cytokines which then play a role in initiating inflammation in the early stages of T2D and they are also known to increase with the progression of the disease (Hotamisligil *et al.*, 1995). Interestingly, more studies have reported on the critical role of innate lymphoid cells in the development of both T2D and cardiovascular diseases (Guarner and Rubio-Ruiz, 2014; Mxinwa *et al.*, 2019).

Elevated levels of circulating inflammatory markers such as CRP and IL-6 are predictive of the development of T2D (Schmidt *et al.*, 1999; Marc Y. Donath and Shoelson, 2011). It has been unclear how the notion of innate immunity and chronic inflammation are involved in the pathogenesis of T2D. However, the role of the innate immunity-related inflammatory pathway in the etiology of T2D has been previously identified (Badawi *et al.*, 2010). According to Arti, Nehal, and Muredach (2011), stimulation of the innate immune processes within adipose tissue is thought to be associated with insulin resistance induced by obesity (Arti, Nehal, and Muredach, 2011). Understanding of the innate immune system offers a new model for T2D and metabolic syndrome pathogenesis, to provide opportunities for the development of novel approaches for this disease prevention in the general population (Ricardo, 2012).

2.3.2. The adaptive immune system

The adaptive immune system, known as the acquired immune system, is a much slower subsystem of the overall immune system. It comprises of very specialized systemic cells and processes which eliminate pathogens. However, it produces a rapid and robust protective response on a subsequent encounter with the same antigen (Bonilla and Oettgen, 2010; Harvard, 2016). The adaptive immune system's three primary functions are to identify non-self, respond to non-self, and remember non-self with its immunological memory (Ricardo, 2012; Ratajczak et al., 2018). Literature shows that adaptive immunity possesses a well-regulated mechanism to facilitate pathogen-specific immunologic effector pathways using T and B lymphocytes (Bonilla and Oettgen, 2010). In addition, sets of gene segments are rearranged during the development of the T and B cells. These are assembled to create genes encoding the specific T and B lymphocyte antigen receptors (See figure 2) (Chaplin, 2010). Furthermore, the adaptive immune response relies on the receptors selected and which persist for life in the host (Ricardo, 2012; Sakaguchi *et al.*, 2008).

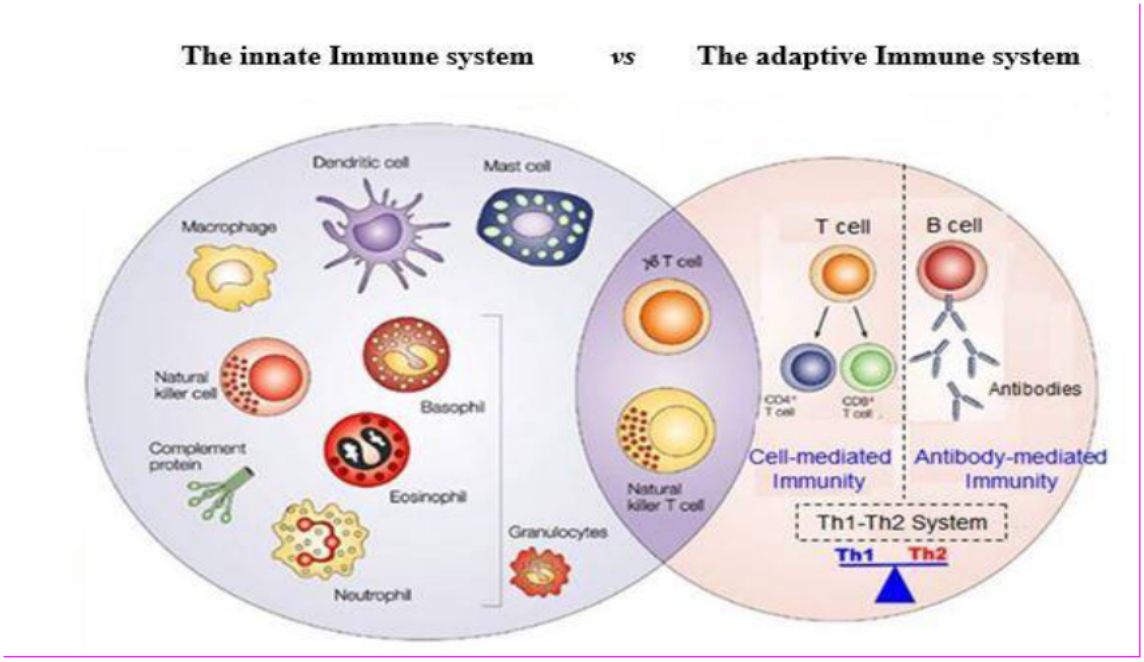

Figure 2. *The innate immune system and the adaptive immune system*

(Adapted from McCulley, D. 2015, https://www.deathtodiabetes.com/reverse-autoimmune-diseases-wellness-program.php).

The innate immune system comprises phagocytic cells that release inflammatory mediators, whereas the adaptive immune system creates a pool of antigen-specific T and B cells capable of producing memory cells that activate a rapid immune response after subsequent exposure to the same antigen.

Pattern recognition receptors (PRRs) are the key initiators of inflammatory responses on the cell surface and control both innate and adaptive immunity activation (Chaplin, 2010). Toll-like receptors (TLRs) are also activated in fat tissues, thereby increasing the inflammatory response (Cruz, Fernandes, and Gomes, 2012). Hence a link between adipose mass and the adaptive immune response has been noted as a relationship that contributes to obesity-associated diseases like T2D (Schuster, 2010). Another study concluded that the adaptive immune system might also contribute to obesity-induced inflammation in conjunction with early insulin resistance by modification of the number and the activation state of adipose tissue macrophages (Esser *et al.*, 2014).

Studies have reported that adipose expansion is correlated to insulin resistance and the release of inflammatory cytokines such as TNF-a, IL-6. High levels of inflammatory factors, such as PAI-

1, CRP, and monocytes activation, lead to T2D (Donath, 2014). Though the adaptive immune system plays a critical role that has been portrayed as driving inflammation in T2D and promoting insulin resistance, the underlying mechanism is still unclear. The adaptive immunity is vital for glucose homeostasis and the control of adipose tissue inflammation in obesity and T2D (Esser *et al.*, 2014). In recent studies, it has been suggested that the adaptive immune system, especially T lymphocytes play a crucial role in the pathogenesis of T2D (Xia, Rao, and Zhong, 2017; Jia *et al.*, 2016; Xia, Rao and Zhong, 2017).

2.4. The Inflammatory Response in type 2 diabetes

Inflammation enables harmful stimuli to be absorbed, thus encouraging wound healing and maintaining healthy homeostasis of tissue (Ahmed, 2011). It has been reported that mononuclear cells such as macrophages, lymphocytes infiltrate into tissue injury sites during systemic inflammation (Harford *et al.*, 2011). Furthermore, two stages of inflammation have been noted, the acute inflammatory phase (i.e., the initial phase) and the chronic inflammatory phase, which occurs if the stimuli persist (Vincenzo *et al.*, 2015).

Previous studies have demonstrated that the disruption of tissue homeostasis stimulates the activation of macrophages, mast cells, cytokines, and chemokines, recruiting leukocytes from circulation into tissue damaged sites (Amir-Zilberstein *et al.*, 2007). Conditions like obesity may lead to adipose tissue hypoxia, insulin resistance, and inflammatory response, thus linking inflammation to the insulin resistance caused by obesity and T2D (Carmeliet, 2005). In T2D, the inflammation process is unregulated and chronic, which leads to chronic immune activation, exhaustion, and, ultimately, immune dysfunction (Pickup, 2004; Schietinger and Greenberg, 2014). Knowledge and understanding of the inflammatory mechanism can assist in addressing specific treatments in inflammatory disorders, thus preventing disease progression.

2.4.1. The Acute inflammatory phase

T2D is often linked to an acute-phase reaction which mediates low-grade inflammation (Pickup and Crook, 1998). Previous studies have reported that trans-membrane receptors called pattern-recognition receptors (PRRs) recognize inflammatory stimuli (Badawi *et al.*, 2010; Moro-garcía *et al.*, 2018). Furthermore, PRRs recognize pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). It initiates an acute inflammatory response with the subsequent release of TNF, IL-1β, and IL-6 pro-inflammatory cytokines (Ahmed, 2011).

Some studies have reported upregulation in T2D patients of CRP, α1-acid glycoprotein, serum amyloid A (SAA), plasminogen activator inhibitor-1 (PAI-1), and haptoglobin, the primary markers of acute-phase response (Shoelson, Herrero and Naaz, 2007; Grossmannm *et al.*, 2015). Other researchers have documented high concentrations of these markers, together with cytokines and chemokines circulating in patients with T2D (Dalla Vestra, 2005; Grossmannm *et al.*, 2015).

2.4.2. The Chronic inflammatory phase

The chronic inflammatory response is well organized and is an important part of the mechanism of human defense against pathogens (Donath and Shoelson, 2011). Some studies have demonstrated chronic low-level inflammation of the insulin action tissues to be linked with obesity-related conditions like diabetes mellitus and insulin resistance (Lontchi-Yimagou *et al.*, 2013).

Previous studies have shown that adipose tissue that is growing rapidly expands faster than the vasculature that maintains the oxygen demand that induces hypoxia. This results in the accumulation of macrophages, thus activating pro-inflammatory cytokines (Lontchi-Yimagou *et al.*, 2013; Weisberg *et al.*, 2003; Xu *et al.*, 2003; Donath and Shoelson, 2011; Harford *et al.*, 2011). Recent studies have also shown that obesity in adipose tissue causes chronic local inflammation. Macrophages are instrumental in inflammation of adipose and systemic metabolic abnormalities (Han and Levings, 2013).

Other studies have reported a significant number of inflammatory factors such as IL-1β, TNF, and CCL2 that are elevated in obesity. The macrophage ratio in adipose tissue is correlated to the extent of obesity (Esser *et al.*, 2014; Donath, 2014). Several other studies have also reported on the elevated levels of Interleukin (IL)-16, Proteins such as CRP and fibrinogen circulating in the acute phase in the liver which is the leading site of IL-6 induced CRP production in obesity-induced insulin-resistant individuals (Pradhan *et al.*, 2001; Spranger *et al.*, 2003; Herder *et al.*, 2009; Schuster, 2010). Studies stated that the existence of TNF in adipose tissue shows evidence of tissue inflammation in T2D and insulin resistance pathogenesis (Donath and Shoelson, 2011; Riddy *et al.*, 2018).

T cell dysfunction results from prolonged exposure to pro-inflammatory cytokines such as TNF-α, which dissociates the TCR signal transduction paths by inhibiting the TCR / CD3 complex's assembly and stability (Ali *et al.*, 2001; Goronzy and Weyand, 2001; Cope, 2002). Besides, prolonged immune activation ultimately leads to T cell exhaustion and AICD (Barber *et al.*, 2006). This suggests that antigen-independent, cytokine-dependent pathways drive T-

cell survival and effector responses. (Cope, 2002). It may be beneficial for therapeutic uses to restore T cell homeostasis (Ali *et al.*, 2001; Goronzy and Weyand, 2001; Cope, 2002).

2.4.3. Adipose tissue inflammation in obesity

Obesity is classified as a Body Mass Index (BMI) of 30 and above, characterized by an enlarged tissue mass divided into two classes of white adipose tissue (WAT) and brown adipose tissue (BAT) (Cooke *et al.*, 2016; Burke *et al.*, 2017). Obesity is also thought to be a chronic inflammatory condition characterized by irregular cytokine production, elevated plasma concentration levels of inhibitor-1 plasminogen activator (PAI-1), IL-6, and CRP(Weisberg *et al.*, 2003). It has been observed that the global increase in the incidence of obesity contributes to the increased prevalence of the metabolic syndrome with dyslipidemia, hypertension, and insulin resistance (Koca, 2017; Cooke *et al.*, 2016). Furthermore, chronic low-grade inflammation associated with obesity increases insulin resistance, which is progressing to T2D (Lontchi-Yimagou *et al.*, 2013). The capacity of adipocytes and hepatocytes to react to insulin and trigger glucose absorption is impaired by insulin resistance, hence stimulating macrophages to secrete excessive amounts of the IL-6, TNF-α and other inflammatory mediators (Herder *et al.*, 2007; Cruz *et al.*, 2013).

Previous studies have demonstrated that adipose tissue becomes hypoxic due to rapid expansion in the pancreas, skeletal muscle and the liver of obese individuals (Zeyda and Stulnig, 2009; Esser *et al.*, 2014). It allows ceramide, free fatty acids, diacylglycerol, and acyl-CoAs to serve as signaling molecules that stimulate inflammation due to the resistance of insulin (Shoelson, Lee, and Goldfine, 2006). Studies show that adipose tissue infiltration by macrophages occurs, which is linked with obesity, low-grade inflammation, and T2D (Donath and Shoelson, 2011).

Studies show that obesity is regulated by Leptin, which is an adipose tissue-derived hormone that has been related to several metabolic inflammations. Homeostatic factors and its serum concentration is directly proportional to the amount of adipose tissue (Esser *et al.*, 2014).

2.4.4. Chronic Inflammation and T cell activation

CD4 + T cells play a significant role in chronic inflammatory disease pathogenesis and are gained as a result of extended exposure to pro-inflammatory cytokines like tumor necrosis factor (TNF)-α (Cope, 2002). It has also been shown that antigen-independent, cytokine-dependent mechanisms drive T-cell survival and effector responses, and potential therapeutic approaches are aimed at restoring T-cell homeostasis to avoid and control metabolic diseases (Avni and Rao, 2000).

Research showed that the transcriptional competence of T cells happens at various stages that are: initiation phase, interaction phase, and acute gene transcription phase. Also, naïve T cells are activated by MHC/peptide complexes during the initiation process via their T-cell receptors (TCRs), where differentiation of those that form a functional immunological synapse occurs. (Cope, 2002)

The interaction of TCR/CD3 complexes with cytokine receptors occurs after engagement with cytokines, and production of IL-2 is stimulated, however only a few T cells enter the cell cycle after this early stage of maturation (Wallin *et al.*, 2001). The induction and recruitment of Th-subset unique transcription factors take place during the engagement process (Szabo *et al.*, 2000). Eventually, secondary interaction with the antigen in the acute gene transcription process includes the mobilization of the nuclear factor of activated T cells (NFAT) along with subset-specific transcription factors into the transcriptosome complex (Sakaguchi *et al.*, 2009). Firm commitment to a particular lineage happens progressively in regional lymph nodes, where the expression and stability of the TCR on the T-cell surface, its avidity for MHC/peptide complexes, the signal strength, and the integrity and signaling adaptor molecules would be important in assessing both qualitative and quantitative qualities of the immune response (Cope, 2002).

Studies have shown that CD4 + T cells separate into Th1 cell subsets and generate IFN-ÿ while others are IL-4, IL-5, and IL-13 Th2 cells (Moro-garcía *et al.*, 2018). The events regulating T-cell effector responses in the context of chronic immunoinflammatory responses, however, remain unclear (Moro-garcía *et al.*, 2018). Most notably, cytokine dysregulation contributes to chronic inflammation, which is characterized by T-cell activation and elevated expression of T-cell surface receptor markers, including CD69, CD38, and HLA-DR (Daniels and Teixeiro, 2015). Besides, other studies also demonstrated that the secretion of pro-inflammatory cytokines IL-2, IL-1β, IL-17, TNF-α and the activation of $CD4^+$ T-cells due to hyperglycemia triggers the NF-kB pathway which leads to "T cell" activation and T2D (Isomaki *et al.*, 2005; Kim *et al.*, 2015). The aforementioned studies show that hyperglycemia stimulates the production of proinflammatory cytokines that ultimately lead to chronic inflammation in patients with T2D inducing chronic T cell activation. In comparison, a previous study reported that hyperglycemia inhibits T-cell activation by blocking the calcium (Ca2+) signal ligand-binding process in Jurkat cells, as Jurkat cells suppress the expression of a T cell receptor (Pavón *et al.*, 2013).

Immune dysfunction would lead not just deficient immunity and inability to produce successful Th effector responses, but to a lack of tolerance due to impaired TCR-induced expression of FasL (Fas ligand), amplification of activation-induced apoptosis, and inability to build

substantial immunoregulatory reactions. It was reported that T-cell normalization is restored following treatment with remission-inducing therapy due to the failure of the adaptive immune system to control an inflammatory response (Isomaki *et al.*, 2005; Kim *et al.*, 2015). This strengthens the need and importance of this research to examine T-cell activation markers and contribute to existing information on how the inflammatory process could promote the development of T2D, which would encourage earlier diagnosis, prevention, and treatment of T2D and other metabolic disorders (Pavón *et al.*, 2013).

2.5. T cell activation in the pathogenesis of Type 2 diabetes

T lymphocytes have been shown to play a pivotal role in T2D pathogenesis. Previous studies have shown interest in understanding the function of T cells in T2D pathogenesis and, in particular, the effects of regulatory T cells on chronic inflammation (Yuan et al., 2018; Rattik et al., 2019).

2.5.1. T cell activation and co-stimulatory receptors

Activated T cells have been noted to play a critical role in the immune response, facilitated by the co-stimulatory receptor CD28 by controlling the activation of naïve and memory T cells by an antigen-presenting cell (APC) (Goronzy and Weyand, 2008). Besides, the activation of T cells depends on the level of the signal obtained by the T-cell receptor (TCR) and on the signals generated by the co-stimulating molecules (Harford *et al.*, 2011; Xia, Rao and Zhong, 2017). Previous research has shown that T cells are equally capable of detecting co-stimulatory and co-inhibitory signals from the environment, and there is a convergence of positive and negative signals during activation (Leibson, 2004). Activation of the innate immune system resulting from macrophage infiltration has been associated with risks related to obesity in T2D (Shoelson, Herrero, and Naaz, 2007).

Studies have shown that co-stimulating molecules are responsible for controlling the initial stages of an immune response while naïve T cells recognize an antigen, and its signals correlate with TCR-mediated peptide identification (Murphy, Nelson and Šedý, 2006). Other studies have portrayed that co-stimulatory molecules which mediate the communication between T cells and APC trigger an irregular immune response that results in loss of pancreatic β cells and tolerance to insulin in T2D (Bouloumie *et al.*, 2005; Zhu, Yao, and Chen, 2011).

It was indicated that the primary signal needed for the activation of T cells is mediated by the TCR, which acknowledges the antigenic peptide within the MHC context. TCR activation alone, however, does not induce a positive-cell response but energizes T cells (Daniels and

Teixeiro, 2015). A secondary signal is reflected in the APC and necessitates an optimum response from T cells (Esser *et al.*, 2014). B cells have also been noted to work as APCs and can stimulate T cells through their classical stimulatory pathway CD28-CD80/CD86 (Goronzy and Weyand, 2008). Furthermore, the upregulation of CD28 and its subset PD-1 is closely linked to the severity of T2D patients with diabetic atherosclerotic macrovascular diseases (Shi *et al.*, 2013). Increased levels of CD4+CD28-T cells in T2D patients with proliferative diabetic retinopathy have been reported (Canton *et al.*, 2004). By developing IL17, this group of co-stimulatory cells was involved in the intensity of T2D (Phoksawat *et al.*, 2016).

2.5.1.1. TCR- T cell receptor

The TCR complex is unique to all T cell clones. This contains subunits for the disulfide-linked recognition of α and β antigen. It is engaged in the process of transmitting signals across the plasma membrane in three types of processes, which are aggregation, conformation shift, and TCR segregation (Van Der Merwe and Dushek, 2011). Also, T-cell activation takes place when a T-cell faces an antigen on an antigen-presenting cell (APC) that is regulated by TCR involvement with the peptide antigen displayed on MHC molecules (except for γδ T cells and NK cells). T cells, however, need many different signals to activate enough to withstand proliferation and differentiation (Lanzavecchia, 2013; Goronzy and Weyand, 2008; Harford *et al.*, 2011).

Previous studies suggested that TCR engagement occurs when TCR binds to a molecule/peptide complex of self-MHC to activate a signal. This results in alterations in cytoplasmic portions of CD3, including phosphorylation of residues of tyrosine. We are also known as TCR triggering, and the self-peptide MHC complexes cooperate with foreign peptide-MHC complexes, which enable the T cell to differentiate, and no signals result in apoptosis (Lanzavecchia, 2013; Isomaki *et al.*, 2005; Van Der Merwe and Dushek, 2011). TCR signaling plays an important role in the adaptive immune response resulting in the expression of the CD28 family, such as the inducible costimulatory molecule (ICOS) and programmed cell death (PD-1), that also plays a powerful role in impairing TCR signaling, comparable to CTLA-4 (Jain *et al.*, 2010).

Some studies show that alterations in TCR (TCR revision) may occur, which activates the development of auto-aggressive T cells that mediate autoreactive pancreatic β-cell destruction. Furthermore, this leads to hyperglycemia after insulin loss; however, a better understanding of

the interplay between T cell receptor (TCR) and insulin activity is of vital clinical significance usually noted in T1D (Alpdogan, Van Den Brink and Brink, 2012; Tober *et al.*, 2018).

2.5.1.2. The inducible T-cell co-stimulatory (ICOS)

A previous study discovered that the inducible T-cell costimulatory (ICOS) is expressed on both CD4 and CD8 T-cells and belongs to the CD28 family. It also acts by stimulating the proliferation of T-cells and T-cell-dependent antibody responses as well as the development of many cytokines such as IL-4, IL-5, IL-10, INF- γ, and TNF-α (Coyle *et al.*, 2000; Y. Zhang *et al.*, 2016). ICOS is not displayed on resting or naïve T cells, but ICOS will induce naïve CD4 + T cells to distinguish and stimulate Th17 cells instead of Th1 cells where they are easily upregulated after TCR involvement (Hutloff *et al.*, 1999; Coyle *et al.*, 2000; Yamada, Salama, and Sayegh, 2002). It has also been reported that ICOS/ inducible T-cell costimulatory ligand (ICOSL) plays an integral part in various immune responses mediated by diseases. Yet ICOS / ICOSL's specific role in the pathogenesis of T2D remains unclear (Khan *et al.*, 2018).

Another research found that the expression of ICOS on T cells is strongly associated with that of CTLA-4 and PD-1, which is regulated by both TCR and CD28 signals (Zhang *et al.*, 2016). Several studies show that instead of interacting with B7.1 or B7.2, ICOS binds B7h (ICOSL), which is a homologated molecule to B7.1 and B7.2 (Yoshinaga *et al.*, 1999; Freeman *et al.*, 2000). B7h (ICOSL) is displayed on B cells, dendritic cells, and macrophages. It has also been demonstrated to induce and promote the proliferation and differentiation of B and T cells (Aicher *et al.*, 2000; Ling *et al.*, 2000). It also has been suspected that, as ICOS/ICOSL is upregulated in T cell inflammatory responses, this may be one of the possible mechanisms that may lead to the development of cardiovascular complications in T2DM patients (Khan *et al.*, 2018). Furthermore, the inhibition of high glucose (HG) or advanced glycation end products (AGEs) activation of T cell inflammatory response was reported when the ICOS/ICOSL pathway was blocked thus confirming that the key role of the ICOS/ICOSL pathway is inducing cytokines secretion (Khan *et al.*, 2018). Overall the expression of ICOS/ICOSL in diabetic patients confirms its role in T2DM-associated inflammation together with endothelial dysfunction, which may provide a novel direction towards the treatment of T2D.

2.5.2. T cell activation markers in type 2 diabetes

To activate, proliferate, and differentiate T cell subsets, a series of complex and coordinated immune cell signals are required (Zhang *et al.*, 2016). Several studies have demonstrated that factors such as obesity may be responsible for immune dysregulation by inducing

hyperglycemia, and this can be measured by investigating expression levels of T cell surface activation markers such as CD95, CD38, and HLA-DR to determine the severity of immune dysfunction (Pickup, 2004).

Previous studies showed that obesity increases the expression of MHC class II on adipocytes; therefore, activating CD4+ cells and initiating tissue inflammation. Pro-inflammatory CD4+ subsets (Th1 and Th17) release cytokines, and this further contributes to the pro-inflammatory environment (Nyambuya *et al.,* 2018; Defuria *et al.,* 2013). Other studies, by comparison, indicate that T2D hyperglycemia inhibits T-cell activation by interrupting calcium transduction in MHC signaling (Guy *et al.,* 2013).

Several other studies have used the combination of CD38 and HLA-DR as activation markers of inflammatory conditions (Deeks *et al.*, 2018; Sachdeva *et al.*, 2010). CD69 was the first antigen to be expressed on activated cells and HLA-DR at the latest (Deeks *et al.*, 2018; Sachdeva *et al.*, 2010). There were significant differences in the expression of T-cell activation antigens between normoglycemic and hyperglycemic individuals. However, other studies found no correlations between T-cell antigen expression and glucose metabolism (Nyambuya *et al.,* 2018). It has been noted that a disruption in the balance of T-cell activation and inhibition could lead to T-cell exhaustion (Nyambuya *et al.,* 2018). Another study reported a decrease in CD95 on $CD4^+$ T cells in both the normoglycemic and hyperglycemic groups which may have occurred as a result of a deficiency of co-stimulatory molecules or the up-regulation of negative regulators such as Fas and PD-1 (Nyambuya *et al.*, 2018; Dieterlen *et al.*, 2014).

Many studies typically use the T-cell evaluation multi-parameter flow cytometry assay as this enables the identification of cells that participate in the immune response and provides a unique opportunity to understand the T-cell subsets are triggered by different stimuli (Krishnaswamy, 2015; Habicht *et al.*, 2007; Zhong *et al.*, 2014). It has been shown that the positive correlation between specific alleles encoded within the region of MHC class II (HLA DR) and the development of T2D have produced the best indications that CD4 + T cells play a role in the pathogenesis of this chronic inflammatory disease.

2.5.2.1. CD69 expression on T cells in type 2 diabetes

T cell activation marker CD69 is a lymphoid activation antigen that has been recorded for lymphocyte proliferation in several studies and also acts as a signal transmitting receptor in T lymphocyte analysis (Radulovic and Niess, 2015). It is depicted as a rapid and sensitive marker also used to detect T-cell activation early, and is typically expressed in low levels of CD4+ and

CD8 + T-cells resting (Radulovic *et al.*, 2013). Measurement of the percentage of T cells expressing CD69 has been reported to reflect the state of the cells following immune activation (Radulovic *et al.*, 2013).

CD69 is typically observable in hyperglycemia for early post-activation, which makes it a valuable marker for quantifying the activation of T-cell and T-cell subsets. Natural killer cells also upregulate CD69 upon activation (Radulovic and Niess, 2015; Cibri, 2017). The activation of lymphocyte signaling pathways promotes CD69 gene transcription, CD69 expression is upregulated in CD8+ T cells in obese visceral adipose tissue consistent with T2D, the reason why CD69 is a good marker for immune activation (Jiang *et al.*, 2014).

2.6. T cell regulation in type 2 diabetes

A sequence of complex and synchronized signals are needed to drive T cell subsets to activate, proliferate, and differentiate. T cell exhaustion takes place when T cell activation is imbalanced by positive co-stimulatory molecules, or negative T cell regulators such as PD-1 and Fas are not up-regulated. (Zhang *et al.*, 2016; Sachdeva *et al.*, 2010; Dieterlen *et al.*, 2014). Regulatory T cells in adipose tissues differ from regulatory T cells in lymph nodes in their ability to overexpress genes that encode molecules associated with leukocyte migration and extravasation alongside very high IL-10 transcript levels (Feuerer *et al.*, 2009; Cipolleta *et al.*, 2015).

Regulatory T cells are vital in the progression of T2D in mice and humans, in addition to being important in the regulation of glucose tolerance, insulin resistance, adipocyte hypertrophy, and body weight (Winer *et al.*, 2009; Guzmán-Flores and Portales-Pérez, 2013). In T2D patients, the levels of regulatory T cells are lower (C Zeng *et al.*, 2012; Guzmán-Flores and Portales-Pérez, 2013). Various microvascular and macrovascular complications associated with T2D have been reported to affect levels of diverse types of regulatory T cells (C Zeng *et al.*, 2012). For instance, the $CD4^{+}CD25^{hi}$ regulatory T cells are lower in T2D patients with microvascular complications than in those with macrovascular complications or no complications. The reduced $CD4^{+}CD25^{hi}$ levels may be attributed to T2D complications emanating from the chronic low-degree activation of innate immunity (Zeng *et al.*, 2012).

2.6.1. Regulatory T cells (Tregs) and Effector T cells (Teffs)

Regulatory T cells (Tregs) are subsets of CD4 + and CD25hi, T cells that express FoxP3 + as an essential transcription factor in their production and function (DeNardo and Coussens, 2007) and Th17 is an illustration of a T eff effector. It is important that a series of complex and

organized signals are produced to instigate Tregs and Teffs activation, proliferation, and differentiation. The function of Tregs has been described to regulate Teffs, Tregs suppress inflammation, and Teffs induce inflammation (Zamani *et al.*, 2016). Treg differentiation is driven by TGF-β and IL-2, which signal through STAT-5. However, TGF-β is only stimulated in the presence of IL-6, and CD69 reduces the stimulation of TGF-β by stimulating IL-6 to reduce STAT5 phosphorylation (Cibri, Cibrián, and Sánchez-Madrid, 2017). Notably, in T2D Tregs (Th1, $CD4^+$ $CD25^{hi}$) have been identified to play a fundamental role in the regulation of body weight, insulin resistance, glucose tolerance, and T2D progression (Winer *et al.*, 2009; Guzmán-Flores and Portales-Pérez, 2013). A previous study demonstrated reduced levels of $CD4^+$ $CD25^{hi}$ Tregs in T2D when they were compared with Th17 and Th1 levels (Zeng *et al.*, 2012). This finding could suggest possible increased cell death or an imbalance of the Bcl-2/Bax index; therefore, immunological homeostasis is critical, as an imbalance can lead to pathological states. Tregs have also been identified as a potential therapeutic tool as they regulate immune responses.

2.6.1.1. Fas signaling pathway

Fas have been defined as a cell surface receptor of tumor necrosis factor (TNF) that stimulates apoptotic signals on activation, and its interaction causes other responses, including proliferation, cell differentiation, and immune response maintenance (Felderhoff-Mueser *et al.*, 2000). Studies have shown that Fas / Fas ligand (FASL) is expressed by activated T cells and is sensitized to autocrine and pancreatic apoptosis mediated by Fas / FasL following repeated antigenic stimulation (Felderhoff-Mueser *et al.*, 2000). It has been shown that the signaling pathway of Fas / FasL and AICD T cells are activated by pro-inflammatory cytokines, IL-1β, IL-2, TNF-α, IL -24, and INF-γ (Paulsen and Janssen, 2011). Stressed adipocytes in T2D patients are implicated in the secretion of the pro-inflammatory cytokine, IL-1β, which is involved in the upregulation of Fas by the pancreatic β cells (Donath and Shoelson, 2011; Itariu and Stulnig, 2014).

Previous studies have shown that diabetic patients have high levels of activated cells expressing $CD39^+$ and CD73, which converts ATP released during an inflammatory process, to adenosine (anti-inflammatory). Furthermore, high glucose concentrations induce the expression of apoptotic receptor FAS and IL-1β production, which both contribute to the glucose-induced impairment of pancreatic β-cell secretory function and apoptosis (Guzman-Flores *et al.*, 2015). Anti-inflammatory functions of adenosine in T2D individuals have been reported (Guzman-

Flores *et al.*, 2015). Adenosine induces a decrease in apoptosis in total lymphocytes and CD8+ lymphocytes by downregulating Fas–FasL indicating a possible mechanism of regulating the inflammatory process present in patients with T2D (Guzman-Flores *et al.*, 2015)

Co-stimulatory and inhibitory molecules such as those in the CD80/CD86/CD28 B7 pathway play a very important role in self-tolerance (Granados *et al.*, 2017). The CD80 and CD86 bind to CD28, an activation receptor, or CTLA-4, which is an inhibitory receptor on T cells (Porciello and Tuosto, 2016). The PD-1 and its ligand PD-L1 also belong to the B7 family (Jin, Ahmed, and Okazaki, 2011). PD-1 has been reported to be expressed on activated T cells, and it inhibits T cell activation after binding to ligand PD-L1 (Latchman *et al.*, 2001). The threshold for T cell activation and the amount of cytokines produced is regulated by the level of PD-1 expression and the extent of engagement of PD-1 to its ligand (Dai *et al.*, 2014).

The CD80/CD86/CD28 B7 co-stimulatory is the most commonly known pathway, where CD80 and CD86 can bind to either an activation (CD28) or inhibitory (CTLA-4) receptor on T cells which determines its functional phenotype (Strome, Zhang, and Strome, 2019). The expression of PD-1 on activated T cells inhibits T cell activation after the binding of PD-1 to PD-L1 (Teufel et al., 2019). Expression of PD-1 and the extent of its engagement to its ligands regulates the threshold for T cell activation together with the number of cytokines produced (Strome, Zhang, and Strome, 2019, Teufel et al., 2019).

2.6.1.2. Programmed cell death (PD-1)

The programmed cell death (PD-1) receptor, also identified as CD279, is a member of the superfamily CD28 that provides negative signals when interacting with its two ligands, PD-L1 and PD-L2, leading in exhausted T-cells being regulated (Jin, Ahmed, and Okazaki, 2011). As an inhibitory protein, PD-1 interferes in self-tolerance by impairing activation of T lymphocytes using a process close to that of CTLA-4 and by reducing activation of phosphatidylinositol-3-kinase (P13 K) (Turner *et al.*, 2014). Studies have shown that signaling PD-1 activates intracellular immuno-receptor tyrosine-based switch motif (ITSM) and tyrosine-based inhibitory motif (ITIM) phosphorylation. This activates SHIP-1 or SHIP-2 that also blocks P13 K pathway activation (Riella *et al.*, 2012; Chinai *et al.*, 2015; Wherry and Kurachi, 2015; Xing & Hogquist, 2012). PD-1 has also been noted to have dual roles in immunological tolerance that is peripheral tolerance induction and maintenance. The main role of PD-1, however, in regulating T-cell exhaustion and suppressing effective T-cell responses (Okazaki and Honjo, 2006; Kao, Oestreich, and Paley, 2012). T cell dysfunction is followed by high levels of

inhibitory molecules such as the PD-1 receptor, generally observed under continuous conditions of T cell activation (Moro-garcía *et al.*, 2018). Other researches have shown that PD-1/PD-L1 blockage has been useful in the immunotherapy treatment of various types of advanced tumors (Sunshine and Taube, 2015).

It was also noted that the T-box transcription factor (T-bet) downregulation is related to higher PD-1 expression by stressed CD8 + T cells in chronic inflammation. T-bet, therefore, can impact PD-1 expression on T cells (Kao, Oestreich, and Paley, 2012). T cell fatigue is followed by a rise in inhibitory expression of molecules such as CTLA-4 and PD-1(Moro-garcía *et al.*, 2018). PD-1 signaling also affects cellular metabolism by impairing glycolysis and by facilitating the oxidation of fatty acids. Together, all of these results mean T cells lose their effector roles and take on a depleted and unstable phenotype to become less proliferative.

PD-1 signals meddle with CD28-mediated P13 K activation and consequently hinder IL-2 production, leading to T lymphocyte anergic status (Parry *et al.*, 2005). PD-1 causes CD8 + T cell apoptosis via the receptor PD-1/PD-L1 and interacts with CD80 to prevent T cell activation (Butte *et al.*, 2007; Haspot *et al.*, 2008). PD-L2 is a second PD-1 ligand inhibiting activation of T-cell cytokine production and proliferation of CD8 + T cells (Latchman *et al.*, 2001; Habicht *et al.*, 2007).

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Taking into consideration the literature on markers of T2D associated with chronic inflammation in prediabetes and the prevention of T2D was compiled for this study entitled: "Evaluation of immune activation and programmed death ligand-1 expression on T cells following a short-term high-fat diet". The aim was to evaluate immune function in prediabetes by assessing differentiation-69 (CD69) cluster expression levels on T cells as immune activation markers, and programmed T cell death ligand-1 (PD-1) levels as immune exhaustion markers. Besides, we further investigated whether low dose aspirin (LDA), Clopidogrel, or a combination of LDA and metformin were effective in preventing CVD's in T2D by ameliorating T cell function. This manuscript was formatted following the *BMC Endocrine disorders* guidelines and submitted to the same Journal. It is under review (**Manuscript number BEND-D-20-00148**).

CHAPTER 3: MANUSCRIPT

Evaluation of immune activation and programmed death ligand-1 expression on T cells following a short-term high-fat diet.

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ABSTRACT

Background: Type 2 Diabetes (T2D) accounts for 90% of diabetic cases due to obesity. Recent evidence points to the association of activated T cells in the pathogenesis of T2D however the immune phenotype of activated T cells in this phenomenon is unclear. Therefore, we aimed to evaluate immune function in prediabetes by evaluating expression levels of cluster of differentiation-69 (CD69) on T cells as immune activation markers and programmed T cell death ligand-1 (PD-1) levels as markers of immune exhaustion. In addition, we further investigated whether low dose aspirin (LDA), Clopidogrel or a combination of LDA and metformin were effective in preventing cardiovascular diseases (CVDs) in T2D by ameliorating T cell function.

Methods: Male C57BL/6 mice (n = 34) at six weeks of age were randomized into two groups, the control low-fat diet-fed (LFD) group and the high-fat diet-fed (HFD) group. Expression levels of CD69 and PD-1 on T cells were compared between these two groups. Furthermore, the HFD-fed mice (n = 18) were randomized into three treatment groups (n = 6/ group); a LDA, a metformin and LDA and a Clopidogrel group. T cell function markers were evaluated using multicolor flow cytometry.

Results: The HFD-fed group demonstrated increased weight gain, elevated glucose levels ($p = 0.008$) and insulin levels ($p = 0.026$) within 2 weeks of diet compared to the LFD-fed group. CD69 expression levels on T cells were reduced in the HFD-fed group when compared to the LFD-fed group ($p = 0.0208$) which is associated to impaired glucose tolerance and increased T cell activation. Post treatment, all treated groups demonstrated elevated levels of CD69 expression, however only the LDA treated group demonstrated a significant reduction in PD-1 expression on T cells when compared to the untreated group ($p = 0.0711$).

Conclusion: T cell dysfunction occurs in prediabetes, activated T cells mediate inflammatory responses in T2D. CD69 attenuates inflammation by modulating T cell egress in T2D. LDA ameliorated T cell activation and suppressed expression of PD-1 demonstrating its protective mechanism against CVD's.

Key words: T lymphocytes, chronic inflammation, type 2 diabetes, low dose aspirin, metformin, Clopidogrel.

BACKGROUND

In developing countries, obese individuals are at high risk of developing type 2 diabetes (T2D) and cardiovascular diseases (CVD's). The Public health Sectors are over-populated by patients, challenged by staff shortages and delays in early disease diagnosis and prevention. Delayed treatment interventions impact negatively on the public resulting in an epidemic of this metabolic disorder. T2D is a metabolic disorder related to insulin resistance, chronic inflammation, and activation of the T cells. T2D is controlled by lifestyle changes and medications such as metformin, low dose aspirin, and Clopidogrel [1, 2]. T cell dysfunction has been implicated in T2D; however, further studies should be done to understand this phenomenon [3, 4].

An evolving body of facts points to the crucial role of activated T-cell-mediated receptors in T2D pathogenesis, with an elevated risk of cardiovascular disease (CVD), especially in obese individuals [5-9]. Findings have indicated that T2D is due to obesity-induced hyperglycemia that leads to insulin resistance, followed by inflammatory responses from T cells [2, 6-8]. Another study indicated that there is no correlation between T-cell activation markers, hyperglycemic, and normoglycemic blood glucose concentrations [3].

Despite all that information, however, the actual immune function in T2D of activated T cells remains unaddressed. To evaluate the role of activated T cells in T2D pathogenesis, further research is needed. CD69 is described as a useful fast and sensitive marker for early detection of activation of T cells [10-12], CD69 was suggested to regulate inflammatory immune responses by preventing T cells from leaking out of the tissue and reducing lymphocyte adhesion at inflammatory sites [11]. CD69 also helps to overcome the inflammation by activating the JAK3/STAT5 signaling pathway to prevent Th17 proliferation [13]. Also, CD69 controls the differentiation of regulatory T-cells (Tregs), interferon-π (IFN-ÿ), interleukin-17(IL-17), and IL-22 secretion. By comparison, T cell exhaustion occurs because of excessive activation of T cells, which is shown by programmed death-1 marker (PD-1) upregulation [14]. PD-1 prevents the activation of T cells and modulates negative costimulatory signals [15]. Multiple studies showed high levels of PD-1 in T2D subjects relative to normoglycemic controls, which is an indication of disease severity and increased risk of thrombotic events [1, 16]. Treatment of T2D includes lifestyle changes and medication such as metformin, Clopidogrel , and low-dose aspirin (LDA), which manages various symptoms[17]. Early markers of T2D enable earlier treatment interventions, therefore preventing T2D complications. T2D-related thrombotic events are avoided by the administration of certain anti-inflammatory drugs like LDA, a combination

of metformin and LDA (Met+Asp), or Clopidogrel [18, 19]. Namely, metformin is used to treat T2D as a first-line oral drug. LDA is T2D's most effective cardioprotective drug.

Nevertheless, It is strongly linked to an elevated risk of bleeding [20, 21]. The dual combination of LDA and Metformin results in improved insulin sensitivity, and it has become the most efficacious treatment of T2D [18]. However when used separately, treatment with LDA and metformin is not successful on T2D. T2D patients show elevated platelet reactivity when treated with Clopidogrel when compared to non-diabetic patients. This drug is used as standard treatment for CVD prevention [19]. Clopidogrel is also known to be more potent than LDA in decreasing the rate of CVD's in diabetic patients [22, 23]. Previous studies in the presence of hyperglycemia did not show T-cell activation and T-cell fatigue markers. The effects of T2D therapy on the activation of T cells and the markers of T cell fatigue are not fully investigated.

This research proposed that diet-induced obesity is causing hyperglycemia and insulin resistance that advances to a prediabetes level. Prediabetes is defined by impaired glucose tolerance and T cell activation, which is involved in T2D pathogenesis. Inflammatory responses are mediated by activated T cells. This study aimed at defining immune markers of early T-cell activation and T-cell exhaustion using a high-fat diet-induced prediabetic C57BL/6 mouse model to assess immune function levels in the high-fat diet-fed (HFD) group compared to the low-fat diet-fed (LFD) mice. This research further aimed to determine which treatment drug will improve T cell function in the mouse model of prediabetes. LDA, metformin, and Clopidogrel were investigated since these drugs have been shown to prevent T2D-related cardiovascular complications.

MATERIALS AND METHODS

Study Design

Ethical clearance was gained through the University of KwaZulu-Natal's Animal Research Ethics Committee AREC/086/016. From The Biomedical Research Unit (BRU), UKZN, We obtained a total of thirty-four male C57BL/6 mice at six weeks of age, were obtained from the Biomedical Research Unit (BRU), UKZN. C57BL/6 strain is a well-characterized model for diet-induced prediabetes, and it created an ideal study model to evaluate markers of T lymphocyte activation and exhaustion in normoglycemic and hyperglycaemic conditions. This research further explored the impact of care in prediabetic mice that were fed an HFD on T lymphocyte activation markers and T cell exhaustion. The mice were kept in a controlled setting

with a 12-hour light / dark cycle (light at 6 am and off at 6 pm), normal room temperature range (23oC -25oC), the humidity of approximately 50%, and free water exposure.

Study procedures

At six weeks of age (n=4) mice were terminated by Halothane inhalation (1.5 percent per kg body weight) before the study experiment to establish immunological and hematological baseline profiles and compare them with (1) LFD-fed mice and (2) untreated HFD-fed mice. Subsequently, the remaining C57BL/6 mice (n = 30) were randomized into two diet groups ensuring that no significant variations existed between the two groups. The controlled low-fat diet group fed (LFD) (n = 7) [D12450J, 10% Kcal derived from fat, (Research Diets, New Brunswick, NJ, USA)] and the high-fat diet-fed group (HFD) (n = 23) [D12492 60% Kcal derived from fat (Research Diets, New Brunswick, NJ, USA)] for 6 weeks.

Experimental procedures

A weekly recording of body weights and plasma glucose levels was done. After six weeks of the diet, an oral glucose tolerance test (OGTT) was also carried out. Comparisons between the prediabetic HFD mice and the control normoglycemic LFD mice were made from the results. More correlations between the two groups, including insulin levels, WBC counts, lymphocyte rate, activation expression levels, and exhaustion markers on T cells. More experimental studies were performed on the prediabetic population, which included evaluating whether treatment can boost T-cell activation in prediabetes and suppress T-cell exhaustion by examining levels of CD69 and PD-1 expression on T-cells in untreated and treated cells. Prediabetic mice (n = 23) were randomly separated into 3 treatment groups (n = 6/group); the LDA (3 mg/kg) the LDA and metformin group (Met + Asp) (13 mg/kg), the Clopidogrel group (0.25 mg/kg) and controls (n = 5) PBS (4 ml/kg) which were orally gavaged once a day for 4 weeks. Expression levels of T cell markers post-treatment were compared between treated and untreated control groups, as shown in Table 2.

Sample collection

Blood samples were obtained weekly at a consistently at 8 am from the dorsal pedal veins to minimize pain and to limit discomfort in mice. They were acclimatized to a restraining device to prevent them from visualizing the procedure conducted and to reduce stress. The mice were also placed under a heat-lamp to increase blood flow, and the area of blood collection was

disinfected with an alcohol swab before blood collection. An approximate volume of 200 µl of blood samples was collected weekly into EDTA, and serum separator tubes (SST) (BD Biosciences, USA) for the evaluation of leukocytes and lymphocyte counts using flow cytometry.

OGTT and body weight measurements

At the three-and-seven week diet, both mice fasted for 16 hours overnight. After a 2 g glucose bolus was taken orally for an oral glucose tolerance test (OGTT), a fasting plasma glucose (FPG) and2-hour glucose was then measured. Blood glucose measurements were also obtained weekly using the Accu-Check active blood glucometer, and the OneTouch ® Select ® handheld glucometer (LifeScan Inc., Milpitas, CA, USA) as directed by the manufacturer using tail prick method at 120 minutes. OGTT was carried out again on the prediabetic group after six weeks of treatment. Fasting plasma insulin measurements (mmol/L) were also obtained using the Thermo Scientific Mouse insulin Elisa kit (Invitrogen, Carlsbad, CA USA) as per manufacturer's instructions. Body weights were measured weekly using a portable electronic balance from the BRU.

Flow cytometry

Data acquisition was performed on the FACS Canto II flow cytometer using the FACS Diva software (BD Bioscience, USA) to measure the percentage of CD69 and PD-1 expression on CD8+ T cells due to their association with the progression of the disease. Voltages for FS/SS photomultiplier tubes were set using unstained T cell suspensions to allow a clear separation of $CD4^+$ and $CD8^+$ T lymphocytes. T lymphocyte isolation was carried out as per the manufacturer's instructions (BD ™ IMag Cell Separation System). A panel of multi-color anti-human monoclonal antibodies: CD69 APC-Cy7 (clone FN 50), PD-1 BDV500 (clone EH 12.1), CD4 Pe Cy7 (clone 5K3) and CD8 APC (clone SK1), (BD Biosciences, New Jersey, USA) were set up to evaluate the levels of T cell immune markers (CD69 and PD-1) of activation and exhaustion. The percentage levels of expression of these markers were measured on the FACS Canto II, using the FACS Diva (Becton Dickinson) software. Briefly, a titrated monoclonal antibody cocktail was prepared, and 2.5 µl was used to stain 50 µl of isolated T cells. At room temperature, this mixture was incubated in the dark for 15 minutes and added and mixed 350 µl of lysis buffer. Another 15 minutes of room temperature incubation was performed in the dark, followed by 350 µl of sheath fluid added. Samples were analyzed immediately. Upon staining, the stained lymphocytes were treated swiftly and gated.

Statistical Analysis of data

The data were analyzed using GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA, USA). Non-parametric data were compared using the Mann-Whitney *U* test, and values were reported as median and interquartile ranges. An unpaired student *t-test* was performed for parametric data and reported as median and 25^{th} – 75^{th} percentiles or mean as well as standard deviation (SD). Furthermore, the one-way analysis of variance (ANOVA) has been used to assess the rate of T cell activation in treatment groups. A paired t-test was used for the results of the OGTT on the same animals' pre- and post-treatment. A Wilcoxon balanced data test was conducted for the non-parametric results. A Spearman rank correlation was used for data correlations between quantitative variables. A p-value < 0.05 was considered as statistically significant.

RESULTS

Baseline characteristics of mice

The C57BL/6 male mice (n = 23) who were fed the HFD demonstrated elevated body weight within two weeks of diet when compared to the baseline measurements (to the LFD group), as tabulated (See table 1). Blood glucose levels increased significantly (p = 0.008), and elevated insulin levels (p = 0.026) were also observed in mice fed with HFD, which is consistent with impaired glucose metabolism, insulin resistance, and prediabetes. The median (25^{th} – 75^{th} percentile) of PD-1 in the prediabetic group was slightly higher 128.3 (110.6 -146.0) than the control group, which was 119.3 (110.5 – 128.0). Nonetheless, when compared between the two groups, these results were not statistically significant (p = 0.4837).

Table 1. *Baseline haematological and immunological parameters*

Characteristics	Control (LFD)	Prediabetic (HFD)	*P* value
No. of mice	7	23	n/a
Age in weeks	10	10	n/a
Weight grams (g)	25.0 ± 2.5	26.0 ± 1.9	0.4300
Glucose levels mg/dL	6.1 (5.4 – 6.9)	8.7 (8.5 – 9.2)	0.0080
AUC mmol/L *120 min	636 (55.9 – 702)	765 (715.5 – 784.5)	0.0320
Insulin conc µlU/ml	4.5 (4.4 – 4.6)	4.8 (4.6 – 8.1)	0.0260
WBC $x10^5$ / µl	5.4 (4.8 – 10.10)	7.5 (4.8 – 8.4)	0.7499
% Lymphocytes	89.2 (87.8 – 90.5)	88.54 (86.5 – 90.5)	0.6064
$CD69^+$ T cells MFI	1264 ± 114.3	1152 ± 102.8	0.0208
$PD\text{-}1^+$ T cells MFI	123.2 (110.5 – 128)	124.6 (110.6 – 146)	0.4837

Significant p values are ($p < 0.05$), MFI (Magnetic fluorescence intensity)

T cell markers in diet-induced prediabetes

The reduced rates of CD69 (mean MFI: 1152 ± 102.8) seen in the prediabetic group compared to the control group (mean MFI: 1264 ± 114.3) may suggest immune suppression of T cells or inhibit proliferation of T cells.

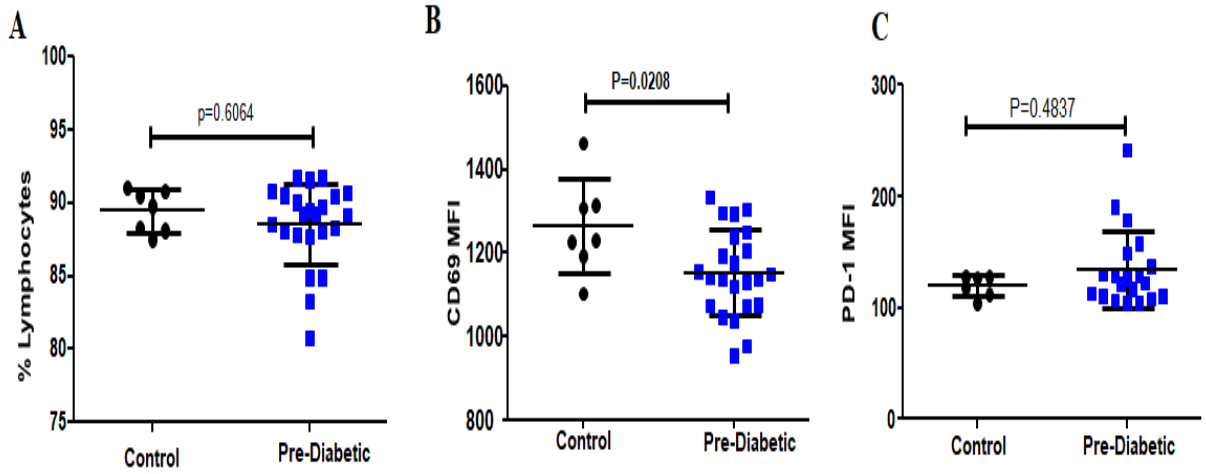

Figure 3. *The response of T cell markers to diet-induced prediabetes.*

(A) Illustrates the percentage of T lymphocytes in the control and prediabetic group (B), the number of CD69 + T cells in both groups, and (C) the number of PD-1+ T cells in each group. The number of T lymphocytes is viewed as a percentage of total lymphocytes, and T cell marker expression was shown as MFI.

T cell markers post-treatment

The expression levels of post-treatment T-cell markers were matched between the treated and untreated control group (see table 2). All treated groups demonstrated elevated CD69 expression levels; LDA ($p = 0.0275$), Clopidogrel ($p = 0.0469$) and Low-dose Aspirin and Metformin (Met + Asp) ($p = 0.0123$).

Table 2. *Expression of T cell markers post-treatment*

T cell Marker	Treatment	Untreated mice (n = 5)	Treated mice (n = 6/group)	*P* value
CD69 MFI	Low-dose Aspirin	1166	1307	**0.0275**
	Clopidogrel	1182	1309	**0.0469**
	Low-dose Aspirin and Metformin	1119	1376	**0.0123**
PD-1 MFI	Low-dose Aspirin	1138	1326	**0.0138**
	Clopidogrel	125.6	119.5	0.4225
	Low-dose Aspirin and Metformin	129.6	119.2	0.5625

Significant p values are highlighted in bold **$p < 0.05$,** MFI (Magnetic fluorescence intensity)

Correlation between T cell markers post-treatment

Significant correlations were observed between the expression levels of T cell markers post-treatment. All treatment groups demonstrated elevated CD69 expression levels compared to untreated HFD-fed control levels. The LDA treated group demonstrated significantly reduced levels of PD-1 ($p = 0.0138$) see Figure 2.

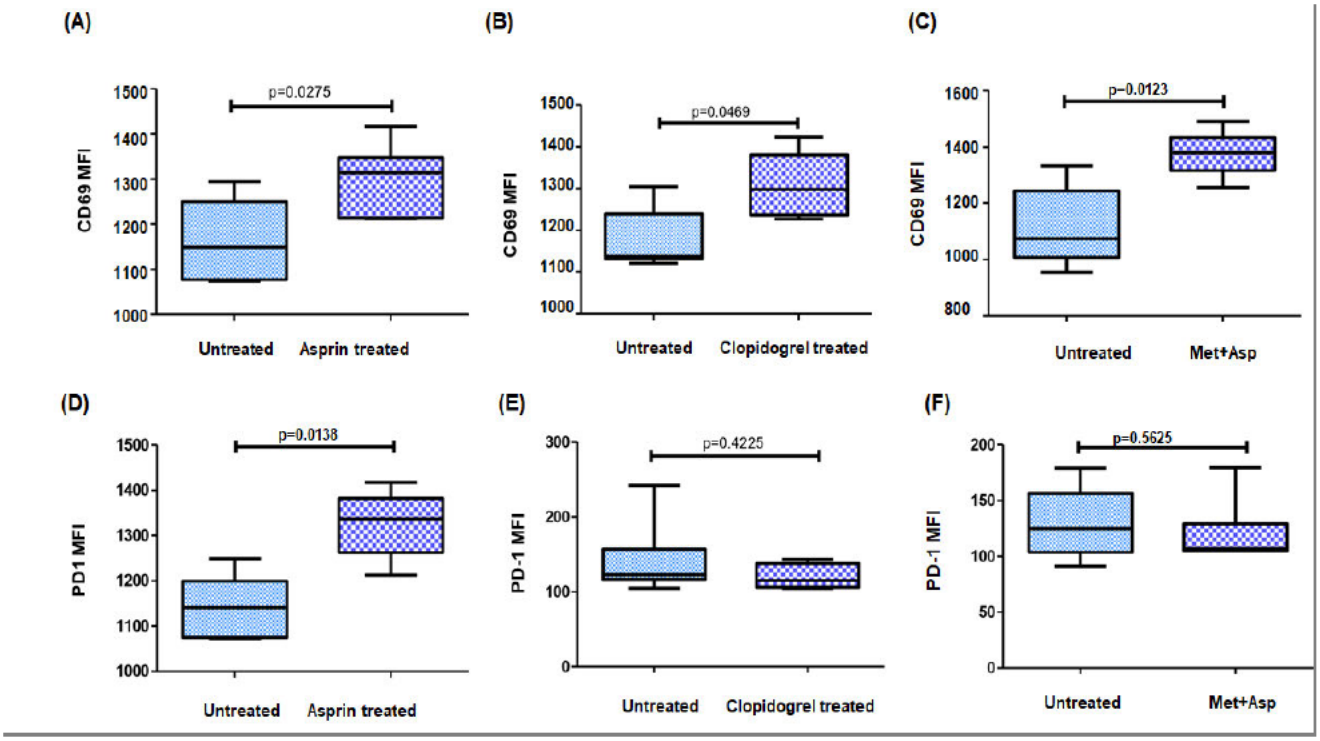

Figure 4. *Expression levels of CD69 and PD-1 markers in treated mice vs. untreated mice.*

(A, B, C) expression levels of CD69 in treated mice vs. untreated mice. (D, E, F) expression levels of PD-1 in treated mice vs. untreated mice. Treatments used on mice (A and D) demonstrates LDA, (B and E) demonstrates Clopidogrel and (C and F) a combination of Metformin and low-dose Aspirin.

DISCUSSION

This study aimed to evaluate levels of T-cell immune activation and T-cell exhaustion in obesity-induced chronic inflammation by assessing CD69 as an indicator of T-cell activation and PD-1 to indicate T-cell exhaustion on C57BL/6 mice after implementation of a High Fat Diet (HFD) as a prediabetes model. We further investigated whether T cell activation is regulated in the chronic inflammation of a prediabetic model of C57BL/6 mice. The C57BL/6 mice given a high-fat diet serve as an excellent model for human prediabetes. The data obtained in this study support our hypothesis that chronic inflammation induces T cell activation and is correlated together with Type 2 diabetes (T2D) with an increased risk of cardiovascular diseases.

The C57BL/6 mice that were fed an HFD demonstrated increased weight gain within two weeks of diet when compared to the LFD group. The OGTT was 765 (715.5 – 784.5) mmol/L in comparison with the control 636 (55.9 – 702) mmol/L with a P-value of 0.032. Glucose intolerance and insulin resistance became evident when the mice presented with hyperglycemia

(elevated blood glucose levels), fasting glucose levels, together with high insulin levels. These findings correlate with findings reported in previous similar studies of high-fat diet-induced hyperglycemia in mice [24-26]. The presence of insulin resistance has been reported to stimulate the release of proinflammatory cytokines IL-6, IFN-γ, and Tumor necrosis factor-α (TNF-α), causing an inflammatory response in the adipose tissue. This study also reported on a significant decline of $CD69^+$ T cells of the HFD group at four weeks of HFD when compared to baseline levels ($p = 0.0208$). This finding is significant because CD69 is commonly described as a rapid and sensitive marker of T cell activation [12, 27]. However, the immune regulatory role of CD69 is under-researched and is not fully understood. CD69 is reported to attenuate inflammatory responses by preventing the egress of Th17 cells by activating the JAK3/STA5 signaling pathway that will inhibit Th17 proliferation.

Furthermore, it also has been reported that CD69 regulates the production of TGF-β and the function of Tregs [12, 28]. This data coincides with reports from previous findings of CD69 being rapidly expressed in elevated levels on T cells upon stimulation and then being reduced in levels of expression as it inhibits lymphocyte proliferation [11]. The significance of this finding is crucial as it will add more knowledge to the under-researched inflammation regulatory role of CD69. With all this being stated, we coincide with the suggestion that threshold levels of CD69 should be used as they are more clinically significant when evaluating CD69 on T cell activation responses [11]. Prolonged activation of T cells results in T cell exhaustion which is normally measured by the measurement of PD-1 expression levels on T cells [29]. This study reported on non-significant findings of the levels of PD-1 expression on T cells even though the PD-1 MFI was higher on the HFD group when compared to the LFD group. This finding could probably suggest that there is no T cell exhaustion present at this stage. Inflammation remains the biggest risk of CVDs and stroke in T2D, which requires treatment drugs that have anti-inflammatory properties [30]. Major adverse cardiac events (MACE) have been reported in T2D patients treated with dual drug combinations of LDA and Clopidogrel . Therefore in our study, we reported on a dual drug combination of Metformin and Aspirin (Met + Asp) as it has been reported to maximize the protective and suppressive mechanism in inflammation preventing CVDs in T2D [18]. Upon introducing the relevant treatments LDA; (Met + Asp); Clopidogrel to the specific HFD groups, our data demonstrated elevated levels of CD69 expression on T cells of all the treatment groups. Low-dose aspirin (acetylsalicylic acid) has previously been reported to have beneficial anti-inflammatory properties by stimulating the upregulation of T regulatory cells (Tregs) and a reduction in glucose and plasma insulin levels is also known, as our data show [21]. The results of this study demonstrated LDA as

the only drug that suppressed levels of PD-1 in HFD group. This is due to its mechanism of stimulating protective Tregs, which contrasts the PD-1 mechanism of inhibiting T cell stimulation. These findings could suggest that the LDA, Met + Asp. Clopidogrel stimulated the Tregs in response to the existing inflammation resulting in the elevated CD69 levels on T cells as it functions as a Treg with anti-inflammatory characteristics. The findings on this study are novel as no other study has demonstrated the dual functions of CD69 collaborated with treatment. This study also successfully demonstrated the effects of LDA, Met + Asp, and Clopidogrel on CD69 while portraying the protective and inflammatory properties of these drugs in diabetes.

Limitations

This study had some limitations, as not all T cell subsets were investigated to determine if there will be differences in their responses. Another limitation is that this study did not introduce Metformin as an individual drug to assess its specific action on T cells in inflammation; therefore, future studies can include this drug. This study will define markers of thrombotic risk for application in resource-limited settings that will facilitate earlier detection of insulin resistance and changes in immune metabolism, which will facilitate access to treatments and prevention of complications in diabetic individuals. This project represents the potential for the development of anti-inflammatory treatment strategies for the prevention of inflammatory-associated thrombosis in chronic inflammatory conditions such as T2D, which is a talented emerging and under-researched area in South Africa.

CONCLUSION

The findings of this study enabled us to prove our hypothesis that diet-induced obesity leads to hyperglycemia and insulin resistance, causing prediabetes. This series of events activates T cells to modulate T-cell mediated inflammatory responses in the pathogenesis of T2D, which may lead to increased risks of CVDs and stroke. LDA was the most effective treatment to resolve inflammation in the prediabetic group treated with LDA. Ameliorated elevated post-treatment CD69 levels demonstrated t cell activity. This resulted in the suppression of PD-1 expression levels due to its action of inhibiting T cell activation. This may suggest that T cell dysfunction is present in prediabetes, and modulation of T cell function is critical at this stage to prevent T2D and eliminate T2D related complications like cardiovascular disorders and increased rate of

thrombotic events. Future studies should perform additional studies on T cell dysfunction in prediabetes to clearly understand the role of T cells in the context of T2D. More studies should also determine the role of CD69 in immune-inflammatory responses to expand on existing knowledge on this phenomenon as there is very limited data on this context. Lastly, other researchers should investigate the mechanism of T2D treatment ion activated T cells to determine the most effective drug in preventing CVD's and stroke.

LIST OF ABBREVIATIONS

APC: Antigen-presenting cell, CD: a cluster of differentiation, CVD: Cardiovascular disease, DM: Diabetes Mellitus, IGT: Impaired glucose tolerance, IL: Interleukin, IFN: Interferon, PD-1: programmed cell death-1, T2D: Type 2 Diabetes, TNF: Tumor necrosis factor, Treg: Regulatory T-cells

DECLARATIONS

Ethics approval and consent to participate

The Animal Research Ethics Committee (AREC) of the University of KwaZulu-Natal, under reference AREC/086/016, approved this study.

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

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Authors' contributions

NM drafted the initial version of the manuscript. BN conceptualized the study and revised the initial draft for its intellectual content and was the major contributor in writing this manuscript. ZM, VM, BN and NM analyzed and interpreted the results. All authors read and approved the final version of the manuscript.

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CHAPTER FOUR: SYNTHESIS – CONCLUSION

4.1. Synthesis and Discussion

Prediabetes is associated with T cell dysfunction which can cause an individual's immune response to be severely compromised, and the complete loss of T cell function can be fatal, therefore studying the mechanisms of how T cells initiate immune responses is crucial to aid in better management and the prevention of this metabolic disorder.

The purpose of this study was to evaluate levels of T-cell activation and T-cell function in the prediabetic state of obese individuals, to establish early manifestation of T-cell dysfunction, and also to establish a possible link between T-cell activity, chronic inflammation, and prediabetes that these individuals describe.

The adaptive immune activity has been tested in most metabolic disorders, but it is not clearly understood as data remains sparse on the immune-phenotype of activated T cells. Work on the immune-phenotype of activated T cells in prediabetes was therefore accomplished by refining a flow cytometry-based assay, which ensured minimal artifact variations and a well-maintained physiological environment of T cells using entire blood. Certain research assessing adaptive immune participation reported inconsistent results due to differences in approached approaches, which lead to a lack of reproducibility.

This study found higher body weight on mice fed the HFD, which was followed by increased levels in the blood and decreased tolerance to oral glucose. Such findings were important as they showed that the mice on a high-fat diet were prediabetic. These results are in conjunction with findings previous researchers reported in prediabetes models (Donath & Shoelson, 2011; Brien et al., 2018). Such findings may indicate that because of the high-fat diet, the mice were in a prediabetic condition.

Besides, after six weeks of the diet, levels of the activation marker CD69 were decreased in the prediabetic group compared with the control group. The interpretation of this finding is that CD69 is widely identified as a rapid and responsive lymphoid activation antigen, widely used to detect T-cell activation early (Martín *et al.,* 2010), however little is known that CD69 has negative regulatory mechanisms as well. In inflammation, CD69 attenuates T cell egress by inhibiting the differentiation of Th17. It further inhibits the phosphorylation of STAT5 that leads to the inhibited proliferation of Th17, therefore, limiting the inflammatory response (Martin *et al.,* 2010). CD69 can also be used for therapeutic benefit as it regulates inflammatory responses (Martin *et al.,* 2010). Another way that CD69 uses to control the egress of T cells to

sites of inflammation is by stimulating the internalization of S1P1, which ultimately reduces the egress of lymphocytes as well (Martín and Sánchez-Madrid, 2011).

Elevated levels of activated T cells correlated directly to the level of immune activation in the prediabetic group. This finding is consistent with that previously described in the literature (Rifa and Widodo, 2014). Furthermore, this finding was also confirmed in previous studies that reported elevated levels of CD69 in hyperglycemia (Stentz and Kitabchi, 2005). The significance of this finding may suggest that activated T cells may be monitored early in the prediabetic state by measurement of this early activation antigen CD69, which will, therefore, assist in preventing disease progression from prediabetes to T2D and enable early treatment interventions in diabetic cases. Therefore, we suggest that T cell activation marker CD69 may be utilized as a valuable and reliable marker in monitoring T cell activation in prediabetic individuals. This observation further confirms the immunological role played by T cells activated in the pathogenesis of T2D.

We further reported on prolonged T cell activation, which resulted in T cell exhaustion. Therefore this activated the up-regulation of negative immune regulators, which caused immune dysfunction. In this study, the diabetic group was reported to have elevated levels of PD-1. PD-1 is an inhibitory protein that works to control T cell exhaustion by inhibiting T cell activation (Jin, Ahmed, and Okazaki, 2011). Increased levels of PD-1 expressing T cells were associated with levels of immune fatigue due to prolonged activation of immune systems.

Furthermore, this finding is compatible with other studies reporting high levels of PD-1 expression in T cells of diabetes associated with diabetic complications (Francisco, Sage, and Sharpe, 2010; Bimin Shi *et al.*, 2013; Zamani *et al.*, 2016). This finding is significant and may be used as an indicator of the severity of T cell dysfunction and the progression of T2D while also preventing complete loss of T cell function. We then propose that PD-1 could be used as a useful T cell fatigue measure as well as a key marker in tracking prediabetes progression to T2D.

We further studied and reported on treatment effects in the prediabetic and T2D classes on activated T cells. T2D therapy used in this study (Metformin, Clopidogrel , and Aspirin) resulted in decreased levels of T cells expressing T cell fatigue marker PD-1 and restored levels of T cells expressing T cell activation marker CD69, thereby enhancing T cell function. Nonetheless, the T2D treatment currently available focuses on improving the glycemic index while neglecting the inflammatory burden; complications may, therefore, occur due to chronic low-grade inflammation.

Our research revealed lower levels of CD69 in type 2 diabetic untreated group when compared to the treated group. This finding was significant and may show that CD69 is an indicator of an ongoing immune response. However, immune exhaustion is described in prolonged untreated T2D. In contrast, the T2D treated group revealed higher levels of CD69, which significantly suggests the regulatory role CD69 plays to regulate immune responses. T cell exhaustion is improved by treatment, and the function of T cells is restored; CD69 levels are therefore elevated due to an increase in activated T cells.

In contrast to the above findings, the T2D untreated group demonstrated higher levels of PD-1 when compared to the treated group. This finding correlated with those reported in other studies that described this finding to be associated with diabetic complications (Francisco, Sage, and Sharpe, 2010; Mcardle *et al*., 2013). The importance of this result may indicate the progression of untreated prediabetes to T2D, which is marked by T cell exhaustion. In further supporting this theory, a previous study reported on a direct correlation between PD-1 and Hs-CRP, which is an inflammatory marker that indicates the gravity of diabetes (Fronczyk A, Moleda P, Safranow K, Piechota W, 2014). Also, lower levels of PD-1 were reported on the treated group, which may significantly suggest that T2D treatment ameliorated T cell exhaustion, and this may also be beneficial in the monitoring of treatment in T2D. This finding inversely correlated with immune activation which further confirmed the regulatory role of PD-1 described in (Jin, Ahmed & Okazaki, 2011). Overall, these findings suggest that early monitoring and treatment of T2D to essential to prevent the loss of T cell function and to preserve the quality of life in obese individuals.

Finally, the findings reported in this study contributes valuable and novel evidence to literature as limited projects have been done on the evaluation of immune function markers in prediabetes. This research further supports the hypothesis that activated T-cells play a crucial role in the chronic inflammatory process that causes prediabetes leading to T2D; however, more studies could continue to investigate and evaluate the function of T cells in the growth of chronic prediabetes inflammation leading to T2D, since data on this work is minimal. T cell investigations are critical as they provide more insight into inflammatory mechanisms and allows for a better understanding of their role, which can greatly assist in addressing specific treatments of inflammatory disorders. Further studies are required to ascertain the value and relevance of increased activated T cells in prediabetes.

4.2. General Conclusion

In conclusion, this study has added an enormous amount of knowledge on the function of activated T cells in T2D. Several factors can induce obesity. However, improper diet and lack of physical activity remain the biggest leading factors. Insulin resistance and chronic inflammation are closely linked to T2D, as well as the activation of T cells, and this suggests that more studies and strategies should be designed to combat this global burden as often, T cell activation is not addressed when designing therapeutic drugs for T2D treatment. T cell-mediated inflammatory responses could be detrimental, as the possibility of mortality is a risk. Investigations of CD69 should be done using threshold measurements as CD69 has different roles in the immune response.

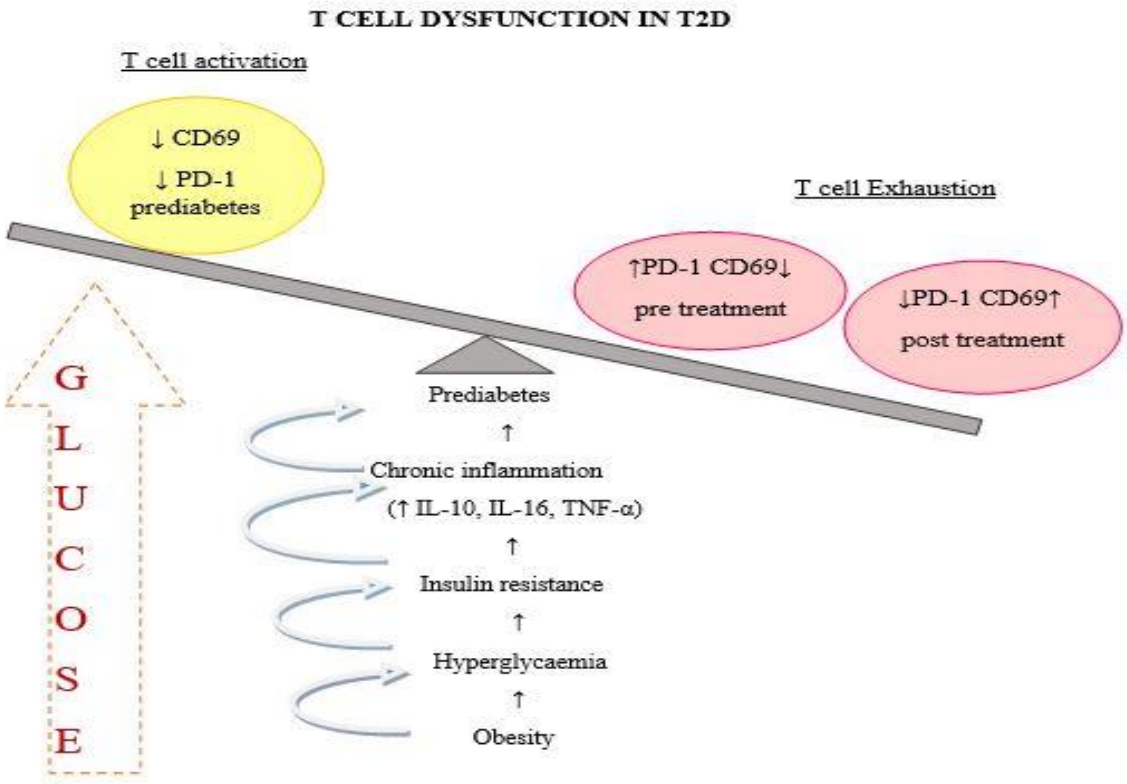

Figure 5. *Dysfunction of T cell in T2D.*

Obesity-induced hyperglycemia causes insulin resistance that triggers the activation of T cells and the release of pro-inflammatory cytokines, including (IL-10, IL-6, TNF-α) inducing a prediabetic state.

Prolonged activated T cell exposure to TNF-α initiates T cell dysfunction and results in low CD69 expression rates on T cells. This may be due to its ability to attenuate T cell egress to inflammation sites and reduce the Th17 differentiation in T cell-mediated inflammatory T2D responses. Treatment with low dose aspirin or a combination of low- dose aspirin or Clopidogrel ameliorates T cell function and modules the T cell-mediated response in T2D, thus preventing T2D related complications such as strokes and cardiovascular diseases.

4.3. Recommendations

Obesity is a major risk factor associated with insulin resistance, and it is also a predisposing factor of T2D. This study was consistent with previous studies that documented elevated glucose levels, chronic inflammation, and dysfunction of T cells in patients with T2D. Not much is known about the activated T cells' role in T2D growth, though. It recommends further investigations as follows:

This study recommends that more studies should be done on T cell immune-phenotype in the pathogenesis of T2D and other inflammatory diseases. This will grow a wealth of knowledge on the function of T cell-mediated inflammatory responses in metabolic disorders. Additionally, other studies should investigate the complex role of CD69 in immune-inflammatory responses due to limited data on the role of CD69. This will help to broaden knowledge base on CD69's actions in inflammatory responses. Finally, researchers can include a broader population to examine the role of activated T cells in T2D pathogenesis and determine the specific drugs that can suppress inflammation to prevent complications such as thrombotic events, CVDs, and stroke.

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APPENDIX

APPENDIX

APPENDIX 1: BIOMEDICAL RESEARCH ETHICS COMMITTEE APPROVAL LETTER

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

16 August 2018

Dr Bongani Brian Nkambule (52541)
School of Laboratory Medicine & Medical Sciences
Westville Campus

Dear Dr Nkambule,

Protocol reference number: AREC/086/016
Project title: Investigating chronic inflammation and immune function in type 2 diabetes

Full Approval – Renewal Application

With regards to your renewal application received on 23 July 2018. The documents submitted have been accepted by the Animal Research Ethics Committee and **FULL APPROVAL** for the protocol has been granted with the following conditions:

CONDITIONS:

- The study must use the collected tissue and blood samples from the study. No additional animals are needed for this study.

Please note: Any Veterinary and Para-Veterinary procedures must be conducted by a SAVC registered VET or SAVC authorized person.

Any alteration/s to the approved research protocol, i.e Title of Project, Location of the Study, Research Approach and Methods must be reviewed and approved through the amendment/modification prior to its implementation. In case you have further queries, please quote the above reference number.

Please note: Research data should be securely stored in the discipline/department for a period of 5 years.

The ethical clearance certificate is only valid for a period of one year from the date of issue. Renewal for the study must be applied for before 16 August 2019.

Attached to the Approval letter is a template of the Progress Report that is required at the end of the study, or when applying for Renewal (whichever comes first). An Adverse Event Reporting form has also been attached in the event of any unanticipated event involving the animals' health / wellbeing.

I take this opportunity of wishing you everything of the best with your study.

Yours faithfully

[redacted]

Prof S Islam, PhD
Chair: Animal Research Ethics Committee

Cc Academic Leader Research: Dr Michelle Gordon
Cc Registrar: Mr Simon Mokoena
Cc NSPCA: Ms Anita Engelbrecht
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