2015

Control and induction of tumor necrosis factor and its receptors on human lymphocytes: a critical structure for immune regulation

https://hdl.handle.net/2144/16138

Boston University
CONTROL AND INDUCTION OF TUMOR NECROSIS FACTOR
AND ITS RECEPTORS ON HUMAN LYMPHOCYTES: A CRITICAL
STRUCTURE FOR IMMUNE REGULATION

by

GEORGES TAHHAN
B.S., University of Florida, 2012

Submitted in partial fulfillment of the
requirements for the degree of
Master of Science
2015
DEDICATION

This work is dedicated to my late grandfather Antonio Mekel, as well as all of those who have been afflicted by diabetes, in hopes to provide some sort of relief to them and their families.
ACKNOWLEDGMENTS

I would like to take this opportunity and thank those who made this study possible. First and foremost, I would like to thank Dr. Denise Faustman for allowing me the chance to continue my involvement in type I diabetes research through her lab. Additionally, I would like to thank Lynn Murphy, Sheila Williams, Doug Burger, and Heather Torrey for all the help and support they provided. Furthermore, I must thank Willem Kuhtreiber, and John Butterworth for being great lab partners, guiding me along the way, and encouraging me to work to the best of my ability. They provided me with countless opportunities for growth in the research community. Everyone in the middle office, Elise Hsu, Danielle Baum, Menghan Zhao, and Sarah Warden, thank you for making this lab such a fun working environment.

I would also like to thank Dr. Liron Abuhatzira for her expert advice in molecular biology, and Dr. Alexandra Rosenfeld for the support provided to continue the real time PCR study. I would like to give a big thanks to the Cutaneous Biology Research Center for their generosity in allowing me to use their top of the line equipment.

Lastly, I would like to thank my family and friends for their unwavering support and encouragement of me reaching my goals. I would not be here without them.
CONTROL AND INDUCTION OF TUMOR NECROSIS FACTOR
AND ITS RECEPTORS ON HUMAN LYMPHOCYTES: A CRITICAL
STRUCTURE FOR IMMUNE REGULATION
GEORGES TAHHAN

ABSTRACT
Type I diabetes (T1D) is an autoimmune disease characterized by the destruction of insulin-producing β cells in the pancreas. Destruction of the body’s own proteins, cells, and tissues is precipitated by the dysfunction of cytokine production, protein modification, and signaling pathways in immune cell subtypes. Tumor Necrosis Factor α (TNFα) and its receptors Tumor Necrosis Factor 1 (TNFR1) also known as p55 and TNFRSF1A, and Tumor Necrosis Factor 2 (TNFR2) also known as P75 and TNFRSF1B play a crucial role in this autoimmune process. TNFα has been shown to stimulate cell death through TNFR1 signaling by the caspase system, while promoting cell survival through TNFR2 signaling using the Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B cells (NF-κB) pathway. Recent findings show a defect in immuno-proteasomes found in autoreactive T cells in people with T1D. This defect causes improper signaling transduction when TNFα binds to TNFR2. The inability to save the cell by activating the NF-κB pathway eventually leads instead to apoptosis using the caspase system. A decrease in TNFα or increase in soluble TNFα receptors might be an explanation for these autoreactive T cells to evade the host immune system, and allow them to cause destruction of the pancreas. We hypothesize that patients with T1D will
show abnormal distribution of TNFα and its receptors at basal levels, as well as when stimulated with interleukins, cytokines, and bacteria such as interleukin-2 (IL-2), lipoteichoic acid (LTA), granulocyte macrophage–colony stimulating factor (GM-CSF), and Bacillus Calmette-Guérin (BCG).

To test this hypothesis, we obtained peripheral blood from T1D patients (n=102) and controls (n=89) and performed in vitro stimulation assays. After a 48-hour incubation, tissue culture supernatants were collected and analyzed for TNF and its receptors production by ELISA, as well as densities of cell membrane receptors by flow cytometry. The data from this study showed significant differences in basal levels of TNFα, TNFR1, and TNFR2 on both the membrane and in the serum between patients and controls. Patients contained a greater percentage of CD4, 8, and 14 – TNFR2 and not TNFR1 double positive cells than their healthy control counterparts. Patient’s sera also contained higher levels of all three markers, sTNFα, sTNFR1, and sTNFR2 than the controls. However, no significant differences were found between patient and controls when stimulated with the various compounds listed above.
TABLE OF CONTENTS

TITLE .........................................................................................................................i
COPYRIGHT PAGE ...................................................................................................ii
READER APPROVAL PAGE ....................................................................................iii
DEDICATION ............................................................................................................. iv
ACKNOWLEDGMENTS ............................................................................................... v
ABSTRACT .................................................................................................................. vi
TABLE OF CONTENTS .............................................................................................. viii
LIST OF TABLES ...................................................................................................... xi
LIST OF FIGURES ..................................................................................................... xii
LIST OF ABBREVIATIONS ....................................................................................... xiv

Chapter 1 INTRODUCTION ...................................................................................... 1
  1.1 The Disease ........................................................................................................ 1
  1.2 Cellular and Molecular Mechanisms of T1D .................................................... 2
  1.3 TNFα and its Role in Autoimmunity .................................................................. 4
  1.4 TNFα Signaling Pathway Through TNFR1 and TNFR2 ................................. 5
  1.4 Immune Regulation Dysfunction ...................................................................... 8
  1.5 Identifying And Quantifying Treg Cells In Peripheral Blood ....................... 8
  1.6 Effects of Specific Cytokines on the Immune System .................................... 9
1.7 Bacille Calmette Guérin and its Benefits .......................................................... 11

1.8 Purpose and Hypothesis .................................................................................... 12

Chapter 2 METHODS .............................................................................................. 13

2.1 Ethics Statement and Study Population ........................................................... 13

2.2 Isolation of PBMC’s From Blood ..................................................................... 14

2.3 Stimulated Tissue Culture Assay ..................................................................... 15

2.4 Flow Cytometry ............................................................................................... 16

2.5 Isolation of Human Serum .............................................................................. 16

2.6 Enzyme-Linked Immuno-Sorbent Assay (ELISA) ........................................... 17

2.7 Isolation of CD4+ T Cells from Blood ............................................................... 17

2.8 DNA Extraction from CD4+ T Cells ................................................................. 18

3.0 Real Time-PCR Quantification of Tregs .......................................................... 19

3.1 Statistical Analyses ......................................................................................... 19

Chapter 3 RESULTS ............................................................................................... 21

3.1 Basal TNFR1 and TNFR2 Expression on Immune Cell Subtype Membranes .... 21

3.2 Basal Levels of TNFα, sTNFR1, and sTNFR2 in Serum ................................. 23

3.3 Effects of Disease Duration on TNFα, sTNFR1, and sTNFR2 Levels in Serum... 25

3.4 TNFR2 Expression on Monocyte Cell Membranes Following Stimulation ...... 27

3.5 TNFα, sTNFR1, and sTNFR2 Concentrations Following Stimulation .......... 28

3.6 Effects of BCG Treatment on Monocytes ....................................................... 30

3.7 Tissue Culture Supernatant Following BCG Treatment ................................. 34

Chapter 4  DISCUSSION ......................................................................................... 35


<table>
<thead>
<tr>
<th>Table</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Study Subject Characteristics</td>
<td>13</td>
</tr>
<tr>
<td>2</td>
<td>Summary of the Data Plotted in Figure 8</td>
<td>24</td>
</tr>
<tr>
<td>3</td>
<td>Summary of P Values Comparing Figure 8 Populations</td>
<td>25</td>
</tr>
<tr>
<td>4</td>
<td>Effects of Disease Duration on TNFα, sTNFR1, and sTNFR2 Serum Levels</td>
<td>26</td>
</tr>
<tr>
<td>5</td>
<td>Effects of Age on TNFα, sTNFR1, and sTNFR2 Serum Levels in Patients</td>
<td>26</td>
</tr>
<tr>
<td>6</td>
<td>Effects of Age on TNFα, sTNFR1, and sTNFR2 Serum Levels in Controls</td>
<td>27</td>
</tr>
<tr>
<td>7</td>
<td>Levels of TNFα, TNFR1, and TNFR2 in Various Diseases</td>
<td>37</td>
</tr>
</tbody>
</table>
### LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Cellular Mechanisms for the Development of T1D.</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>Molecular Activation of TNFα by TACE.</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>TNFα Signaling Pathway Between Normal and Autoreactive T Cells</td>
<td>7</td>
</tr>
<tr>
<td>4</td>
<td>Methylation Specific Real Time-PCR</td>
<td>9</td>
</tr>
<tr>
<td>5</td>
<td>Immune Responses to Pathogens</td>
<td>11</td>
</tr>
<tr>
<td>6</td>
<td>TNFR1 and TNFR2 Surface Expression Profiles</td>
<td>21</td>
</tr>
<tr>
<td>7</td>
<td>Comparison of TNFR1 Expression Between Patients and Controls</td>
<td>22</td>
</tr>
<tr>
<td>8</td>
<td>Comparison of TNFR2 Expression Between Patients and Controls</td>
<td>23</td>
</tr>
<tr>
<td>9</td>
<td>Basal Serum Levels of TNFα, sTNFR1, and sTNFR2</td>
<td>24</td>
</tr>
<tr>
<td>10</td>
<td>Effects of Disease Duration on TNFα, sTNFR1, and sTNFR2 Serum Levels</td>
<td>25</td>
</tr>
<tr>
<td>11</td>
<td>Effects of Age on TNFα, sTNFR1, and sTNFR2 Serum Levels in Patients</td>
<td>26</td>
</tr>
<tr>
<td>12</td>
<td>Effects of Age on TNFα, sTNFR1, and sTNFR2 Serum Levels in Controls</td>
<td>27</td>
</tr>
<tr>
<td>13</td>
<td>Changes in CD14-TNFR2 Positive Cells and TNFR2 MFI</td>
<td>28</td>
</tr>
<tr>
<td>14</td>
<td><em>In Vitro</em> Induction of TNFα, sTNFR1, and sTNFR2</td>
<td>29</td>
</tr>
<tr>
<td>15</td>
<td>Effects of BCG on TNFR2 Expression on Monocytes</td>
<td>31</td>
</tr>
<tr>
<td>Page</td>
<td>Title</td>
<td>Page</td>
</tr>
<tr>
<td>------</td>
<td>----------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>16</td>
<td>Effects of BCG on TNFR1 Expression on Monocytes</td>
<td>32</td>
</tr>
<tr>
<td>17</td>
<td>Effects of BCG on Trans Membrane TNFα Cell Surface Expression on Monocytes</td>
<td>33</td>
</tr>
<tr>
<td>18</td>
<td>sTNFα, sTNFR1, and sTNFR2 Concentrations Following BCG Treatment</td>
<td>34</td>
</tr>
</tbody>
</table>
LIST OF ABBREVIATIONS

AACE .............................................. American Association of Clinical Endocrinologists
ACE ................................................ American College of Endocrinologists
APC ................................................ Antigen Presenting Cell
BCG ................................................ Bacillus Calmette–Guérin
cDC ................................................ Conventional Dendritic Cells
CNS .................................................. Central Nervous System
CSF .................................................. Cerebral Spinal Fluid
ELISA .............................................. Enzyme-Linked Immuno-Sorbent Assay
FITC ............................................... Fluorescein Isothiocyanate
GMCSF ......................................... Granulocyte Macrophage Colony-Stimulating Factor
HBSS .............................................. Hanks Balanced Salt Solution
ICOSL ........................................... Inducible T cell Co-Stimulator Ligand
IDO ............................................... Indoleamine-2,3-Dioxygenase
IFNγ ............................................... Interferon Gamma
IL ..................................................... Interleukin
iNKT .............................................. Invariant Natural Killer T Cells
iPESC .......................................... Immune dysregulation, Polyendocrinopathy, Eteropathy, X-linked
LTA ................................................ Lipotechoic Acid
MFI ............................................... Median Fluorescence Intensity
MS ............................................... Multiple Sclerosis
NF-κB ........................................... Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B cells

NK cell .................................................................................................................. Natural Killer Cells

NO .......................................................................................... Nitric Oxide

PBMC .............................................................................................................. Peripheral Blood Mononuclear Cells

pDC ........................................................................................................... Plasmacytoid Dendritic Cells

PE ............................................................................................................... Phycoerythrin

T1D .............................................................................................................. Type 1 Diabetes

TNFα ................................................................. Tumor Necrosis Factor Alpha

TNFR1 ................................................................. Tumor Necrosis Factor Receptor 1

TNFR2 ................................................................. Tumor Necrosis Factor Receptor 2

Tregs ................................................................. T Regulatory Cell

TSDR ............................................................... Treg Specific Demethylated Region
Chapter 1 - INTRODUCTION

1.1 The Disease

Type I diabetes (T1D) is an autoimmune disease, which causes the destruction of insulin-producing pancreatic β cells in the Islets of Langerhans. These cells are unable to efficiently produce insulin, causing abnormal blood glucose levels, which can lead to micro and macro vascular damage if left untreated.

Several hypotheses have been proposed throughout the years about what factors contribute to the development and progression of T1D. Some of these hypotheses include the ‘accelerator’ and ‘overload’ hypothesis where childhood obesity would increase insulin demand, overloading β cells and accelerating damage [1]. The ‘Copenhagen model’ states that a mixture of the environment, the immune system, and the β cells are the main factors that cause type I diabetes. The ‘hygiene’ hypothesis postulates that reduced exposure to infectious agents prevents proper immune development and can cause a rise of autoimmune disorders [2]. The ‘fertile field’ hypothesis states that microbial infections can cause a state of compromised immune system, allowing for expansion of auto-reactive lymphocytes [3]. Similar to the fertile field hypothesis is the ‘old friends’ hypothesis, which focuses on the role of gut flora in type I diabetes. The ‘threshold’ hypothesis suggests that a mixture of environmental and genetic factors causes type I diabetes. Finally, there is the idea that macrophages in the pancreas produce higher quantity of cytokines, which causes β cells to be phagocytized by antigen presenting cells, which ultimately causes the onset of type I diabetes [4].
This study places heavy emphasis on the immunobiology aspect of the disease. Specifically, how differences in cytokine production such as tumor necrosis factor alpha and its receptors might affect viability and action of damaging autoreactive cells.

1.2 Cellular and Molecular Mechanisms of T1D

Many different kinds of cell subsets contribute to the onset and progression of T1D. The main cell types seen thus far to contribute to T1D are macrophages, dendritic cells, T cells, and natural killer cells [5].

Antigen specific T cells in the pancreatic lymph node can differentiate into either pathogenic effector T cells - also called diabetogenic T cells - or into protective T regulatory (Treg) cells. Since these cells determine the type of immune response to β cell antigens, their balance ultimately determines whether a damaging or a protecting reaction occurs in the pancreas. As seen in Figure 1, there are many cell subsets that play a role in the immunopathology of type I diabetes.

Macrophages are recruited to the islet via CD4+ T cell and β cell secretion of CC-chemokine ligand 1 and 2 [6]. Macrophages are capable of producing interleukin-1β (IL-1β), interleukin-12 (IL-12), Interferon-γ (IFNγ), Tumor Necrosis Factor (TNFα), and Nitric Oxide (NO), which all promote differentiation of diabetogenic CD8+ T cells, and can lead to the destruction of β cells.

Conventional dendritic cells (cDCs) pick up β cell antigens, which then become activated. These activated cDCs travel to draining lymph nodes and prime pathogenic islet antigen specific T cells and macrophages by release of IL-12, a T-cell stimulating
factor. Primed pathogenic islet antigen specific T cells - also called diabetogenic T cells - can then proceed in the destruction of β cells in the pancreas.

However, plasmacytoid dendritic cells (pDCs) and invariant natural killer T (iNKT) cells activate Treg cells through the release of indoleamine-2,3-dioxygenase (IDO), IL-10, TGFβ and inducible T cell co-stimulator ligand (ICOSL). iNKT, pDC, and Treg cells all play an important role in delaying the onset of T1D by inhibiting the actions of diabetogenic T cells through secretions of IL-10, TGFβ, and IDO [7].

**Figure 1. Cellular Mechanisms for the Development of T1D.** After cDC phagocytize β cell antigens, it becomes activated and can either stimulate pathogenic islet antigen specific T cells which contribute to the development of T1D, or stimulate Treg cells which contribute to the protection from T1D (from Lehuen et al.) [7].
1.3 TNFα and its Role in Autoimmunity

TNFα is a key protein of the innate immune system. TNFα is secreted by the host in response to infection by a microorganism, which leads to the inactivation and neutralization of the threat. This innate immune response, of course without the refined features of memory, like the T and B cells, has been around for so long as a first line of protection. Microorganisms have generically evolved in diffuse ways to thwart the TNFα response [8]. Mechanisms to lower biologic TNFα include host shedding of TNFR1 and TNFR2 receptors from cell membranes and many more.

TNFα, aside from being necessary for infection control, also plays an important role for several beneficial immune effects. Tregs require the prescience of TNFα to survive, but exposure of autoreactive T cells in the periphery to this same TNFα causes programed cell death. It has been hypothesized by many that the reason autoimmune diseases and allergies are on the rise, is because there has been the elimination of many infectious diseases. This hypothesis, as stated earlier, is called the Hygiene Hypothesis. We have proposed that TNFα is the central cytokine that is deficient in autoimmune prone households of our clean environment, and deficiencies in this cytokine drive the rising rates of self-reactive diseases.

This hypothesis might seem contradictory since very impressive biologics of anti-TNFα are mainstream therapy for some forms of autoimmunity like rheumatoid arthritis. However, the human data is an important example in that diffuse autoimmune diseases as it relates to possible deficiencies of TNFα causes diverse autoimmune diseases such as type 1 diabetes, multiple sclerosis, celiac disease, and Crohn’s disease.
1.4 TNFα Signaling Pathway Through TNFR1 and TNFR2

TNFα is primarily produced and stored on the surface membrane of immune cells as a stable homotrimer. From this state, TNFα may become activated by proteolytic cleavage by the metalloprotease TNFα converting enzyme (TACE). Once in solution the trimeric 51kD TNFα dissociates into singular 17kD TNFα molecules where it reaches full activation [9].

Figure 2. Molecular Activation of TNFα by TACE. Association of iRhom2 with TACE facilitates its folding and transportation from the endoplasmic reticulum to the golgi and subsequently to the surface membrane where it is then able to proteolytically cleave TNFα. Once TNFα is cleaved, it dissociates from its trimeric state into singular TNFα molecules. At this stage, TNFα can either bind to soluble or membrane bound TNFR1 and TNFR2 (from Beutler et al. 2012) [10].
Once TNFα dissociates from its trimeric form, into its singular form it is considered to be biologically active. TNFα is the natural ligand for both TNFR1 and TNFR2. At this stage, it can either bind soluble or membrane bound TNFR1 and TNFR2. Binding to any of the two soluble TNFα receptors will render TNFα inactive, and will not surmount a signaling response. This concept is considered to be a defense mechanism by invading hosts such as tuberculosis (TB) to evade detection and destruction by the host immune system. The intracellular mycobacterium increases shedding of TNFR2 receptor, which then binds and inactivates TNFα thus shielding itself from destruction [11].

TNFR1 is found ubiquitously throughout every cell in the body, while its counterpart TNFR2 is more restrictively expressed on certain immune subpopulations. Normally, TNFα signaling through TNFR1 pathway will lead to apoptosis through the caspase system. On the other hand TNFα signaling through TNFR2 will lead to cell survival through activation of NF-κB pathway. However, it has been found that autoreactive T Cells have a defective immuno-proteasome which causes the inability to activate the NF-κB pathway when stimulated through TNFR2 [12]. This inability of the autoreactive T Cell causes the cell instead to signal through the caspase pathway leading to apoptosis as seen in Figure 3.
Figure 3. TNFα Signaling Pathway Between Normal and Autoreactive T Cells. TNF binding to normal T Cells will elicit either an apoptotic pathway through TNFR1, or cell survival pathway through TNFR2. However, defects in the autoimmune cell prevent signal transduction through the TNFR2 pathway, thus leading to apoptosis when exposed to TNFα. (from Faustman et al. 2010) [12].

Transmembrane TNFα can be found on a wide array of cell populations such as macrophages, monocytes, neutrophils, natural killer cells (NK cells), and many lymphocytes with exception of CD8+ T cells [13]. The process of secreting this cytokine as described above can be in response to many kinds of stimuli such as IFNγ, IL-2, GM-CSF, and bacterial lipopolysaccharides [14].
1.4 Immune Regulation Dysfunction

Regulatory T cells, also known as Tregs, express forkhead box protein 3 (Foxp3), an intracellular transcription factor that is an identifier of this unique cell population with surface CD4 expression and high levels of the CD25 (IL2 receptor) [15]. It is well appreciated that having sufficient numbers as well as functional Tregs is essential for immune tolerance and the prevention of autoimmunity. Indeed early work identified people and murine models with FoxP3 gene mutations caused fatal autoimmune diseases and allergies [16,17]. The key role of FoxP3 gene in the maintenance of self tolerance was first shown in scurfy mice and subsequently in patients with immune dysregulation, polyendocrinopathy, enteropathy, X-linked (iPeX) syndrome as the causative genetic anomaly that results in severe autoimmune diseases and allergy, which resemble the diseases observed following depletion of CD4+CD25+ Treg cells in rodents [18-20]. In the peripheral blood these cells comprise about 1-4% of the total CD4+ T cell population.

Identifying Tregs, especially human Tregs has been a multi-year challenge for the research community. It would indeed be worthwhile to identify Tregs by more cell surface receptors. TNFR2, unlike its ubiquitous TNFR1 receptor, prominently identifies very potent and very functionally suppressive Tregs. Furthermore, the TNFR2 expression on Tregs is 10-100x higher than TNFR1 expression [21,22].

1.5 Identifying And Quantifying Treg Cells In Peripheral Blood

Recent technological advances have made it possible to use the methylation status of certain genes to distinguish complete cell subtype populations. Activated Treg cells for
example contain a specific unmethylated gene named FoxP3. Constant expression of this transcription factor is required for the Tregs to maintain their immune regulatory function and ensure immune homeostasis [23]. Stable expression of Foxp3 is achieved by epigenetic modification of the Treg-specific demethylated region (TSDR). This enhancer region is methylated in all other types of cells except for the Tregs. Since only activated Tregs have this region unmethylated, it makes it the perfect target for distinguishing CD4+ Tregs from other CD4+ cells.

**Figure 4. Methylation Specific Real Time-PCR.** One set of primers can be used to amplify a certain area of a gene, which can have one of two hydrolysis probes attached. Then ratios of fluorescence between the probe bound to the methylated DNA and the probe bound to the unmethylated DNA could be used to quantify the number of Tregs in the sample (from Kehrmann et al. 2012) [24].

### 1.6 Effects of Specific Cytokines on the Immune System

In this study we focused on the effects of interleukin-2 (IL-2), lipotechoic acid (LTA), granulocyte macrophage colony-stimulating factor (GMCSF), and Bacillus Calmette–Guérin (BCG) on different immune cell subtypes.

IL-2 is secreted by activated CD4+ cells, and has a pleiotropic effect on the
immune system. It increases Foxp3 transcription in Tregs, increasing their proliferation, survival, and suppressive abilities. It also stimulates cytotoxic CD8+ T Cells, as well as T helper cells [25].

The study included LTA as a means of simulating the effects of gram-positive bacteria. BCG for example is one of the many gram-positive bacteria where LTA serves as one of the major cell wall components. Immune cell subtypes such as neutrophil granulocytes, and monocytes exposed to LTA soon acquire typical activated cell morphology. LTA has been shown to increase secretion of proinflammatory cytokines such as interleukin-8, TNFα, and GM-CSF by peripheral blood mononuclear cells (PBMC). The proinflammatory cytokines LTA induces are all associated with NF-κB pathway, and therefore increased life span of the cells [26]. Granulocyte Macrophage-Colony Stimulating Factor has been shown to induce the production of TNFα, facilitate the maturation of monocytes into macrophages, and increases the viability of monocytes during extended periods of in vitro cell culture incubations [27].

Treatment of type 1 diabetic NOD mice with BCG has shown not only to halt the progression of the disease, but also provide a permanent cure [28]. In this study the mice showed a decrease in autoreactive T cells, an increase in Tregs, and regeneration of the pancreas. BCG administered to humans with type I diabetes showed similar but not all effects as seen in mice. BCG like LTA stimulated the immune system to induce the production of TNFα [29].
1.7 Bacille Calmette Guérin and its Benefits

Bacille Calmette Guérin (BCG) is administered in the developing countries as an effective vaccination against the mycobacterium tuberculosis. BCG is considered a potent immunostimulant. Administration of this mycobacterium normally produces a Th1 immune response (Figure 5) [31].

![Figure 5. Immune Responses to Pathogens.](image)

The Th1 response is more effective for intracellular pathogens such as viruses and bacteria, whereas the Th2 response is more appropriate for extracellular pathogens. (from Cohen et al. 2015) [31].

Production and detection of IFNγ is the key characteristic of a Th1 immune response. High IFNγ concentrations activates the bactericidal activities of macrophages, the cell mediated innate immunity via activation of NK cells, and increases specific cytotoxic immunity using T cell interacting with antigen presenting cells (APC) [32].
Most importantly IFNγ stimulates the secretion of TNFα from mainly activated macrophages.

The subsequent effects BCG has on the host’s immune system ideally can be harnessed and used potentially as a treatment for type I diabetes. BCG activates macrophages, which are the main source of TNFα, and also stimulates the release of IFNγ, which also stimulates the release of TNFα. TNFα is absolutely required for T reg cell viability and function. An increase in Treg cell activity might have suppressive effects in several autoimmune disease such as type I diabetes [33].

1.8 Purpose and Hypothesis

The purpose of this study is to compare tumor necrosis factor receptor densities on the membrane, and the rate of shedding of these receptors from specific immune cell subtypes between controls and people with type I diabetes for both basal and stimulated conditions. This study is also interested in observing the functional effects of BCG in respect to TNF, its receptors, and viability of certain immune cell subtypes.

We hypothesize the TID subjects would exhibit increased TNFR1 and TNFR2 both basally and following stimulation compared to healthy controls. We also hypothesize that the presence of BCG would increase the number of active Tregs cells.
Chapter 2 - METHODS

2.1 Ethics Statement and Study Population

Peripheral blood was obtained by venipuncture from patients with type 1 diabetes and healthy controls with full institutional approval from Massachusetts General Hospital. Written informed consent was obtained from all patients or from patient guardians, as appropriate (MGH/Partners Protocol #2001P001379). Four BD vacutainer EDTA 10 mL vials, and 1 BD vacutainer serum 10mL vial (BD, Franklin Lakes, NJ) were collected from each participant. All blood used in these experiments was fresh, and processed within two hours from blood draw.

A total of 191 randomly chosen participants were enrolled in the study. The clinical characteristics of both the type I diabetics and controls used in this study are presented in Table 1.

Table 1. Study Subject Characteristics. Three distinct populations were studied. Their age, duration of disease, and age of onset (if applicable) are listed as means with standard errors.
People with type I diabetes were of ages ranging from 11 to 72 years, and had
disease durations of at least 2 years. All control subjects neither had an autoimmune
disease nor a family history of autoimmune diseases. The non-autoimmune disease
controls were all between 14 and 69 years of age. The diagnosis of type I diabetes was
based on the criteria of the American Association of Clinical Endocrinologists (AACE)
and American College of Endocrinologists (ACE).

### 2.2 Isolation of PBMC’s From Blood

The BD Vacutainers containing EDTA were used for isolation of peripheral blood
mononuclear cells. All PBMC’s were isolated using ficoll-hypaque PLUS gradient (GE
Life Sciences). Blood was carefully layered over the ficoll in a 50 mL falcon tube, for a
final one-to-one ratio of blood to ficoll. The tube was centrifuged at 400 x g for 20
minutes at 4°C. The lymphocyte layer was transferred to a clean 50mL falcon tube, and
was filled to the brim with Hanks Balanced Salt Solution (HBSS) to wash away any
unnecessary platelets and plasma proteins. The tube was centrifuged at 400 x g for 10
minutes at 4°C. The pellet of cells were resuspended with 4 mL lysis buffer, and placed
on ice for 7 minutes. Following lysis, the contents were transferred to a clean 15mL
falcon tube, and washed with HBSS to remove any residual lysis buffer. The tube was
then centrifuged at 400 x g for 10 minutes at 4°C. The pellet of cells was then
resuspended with tissue culture media consisting of 1640 RPMI with 20% fetal bovine
serum and 1% L-Glutamine and 1% Penicillin/Streptomycin. At this time, a 100µL cell
sample was mixed with 100µL Trypan Blue and counted using a hemocytometer. The tube was centrifuged 400 x g for 10 minutes at 4°C and resuspended in tissue culture media to provide a concentration of 5 x 10^6 cells/500µL.

2.3 Stimulated Tissue Culture Assay

After isolating the PBMC, and resuspending the cells to make a concentration of 5 x 10^6 cells/500µL, 500µL was pipetted into each well in a 24-well tissue culture plate. The samples were divided into 5 batches. The first batch of 500µL containing 5 x 10^6 cells received an additional 500µL of tissue culture media, and was not stimulated. This batch served as a comparison to the rest of the stimulated batches. The rest of the batches received, 10,000 units Interleukin-2 (IL-2) (Life Technologies), 10 mcg Lipotechoic Acid (LTA) (Sigma Aldrich), 10 mcg LTA + 100 ng Granulocyte Macrophage Colony-Stimulating Factor (GMCSF), and 1.56 x 10^5 Bacillus Calmette–Guérin (BCG) (Sanofi), respectively. Each stimulated sample contained 5 x 10^6 cells, the specific treatment, and a supplement of tissue culture media for a total volume of 1mL. The 24-well tissue culture plates were incubated for 48 hours at 37°C 5% CO₂ with a cover but no slip.

Following 48 hours incubation, samples were harvested. The contents of each well were transferred to their respective clean 1.5mL eppendorf tubes. The tubes were centrifuged for 6 minutes at 4500 rpm in 4°C. The supernatant was collected and stored in -80°C for future ELISA’s, and the cells were resuspended with 1mL of tissue culture media for analysis using flow cytometry.
2.4 Flow Cytometry

Following 48 hours incubation, 100μl of cells were collected from each well of the 24-well plate and transferred into a 1.5ml eppendorf tube. For cell surface immunostaining, we used fluorescein isothiocyanate (FITC)-, allophycocyanin (APC)-, and phycoerythrin (PE)-conjugated monoclonal antibodies against CD4 (clone RPA-T4)/CD8 (clone HIT8a), TNFR1 (clone 16803)/TNFR2 (clone 22235), and CD14 (clone M5E2) respectively. CD4, CD8, and CD14 antibodies were all from BD Bioscience Pharmingen, Franklin Lakes, NJ. TmTNFα, TNFR1, and TNFR2 antibodies were all from R&D Systems, Minneapolis, MN. All antibodies were from mouse with human reactivity.

10μL of antibody was pipetted into the 100 μL sample of cells, and placed in the dark for incubation in 4°C for 20 minutes. Following incubation, cells were washed with 1mL of tissue culture media, and centrifuged for 6 minutes at 4500 rpm in 4°C. The supernatant was discarded, and cells were resuspended in 300μL. The sample was then transferred to a clean polystyrene flow cytometry tube, and was measured using BD FACSCalibur and the CellQuest acquisition program. The cell count was set to a total of 11,000 events counted and the same channels were used.

2.5 Isolation of Human Serum

The blood collected in the BD vacutainer serum was used for serum isolation. The tube was centrifuged at 400 x g for 15 minutes at 20°C. This separated the blood into two components. The top component containing the serum was transferred into four sterile
2mL cryovials, and stored in -80°C for use in future experimentation and analysis.

2.6 Enzyme-Linked Immuno-Sorbent Assay (ELISA)

TNFα, sTNFR1, and sTNFR2 were detected by standard sandwich ELISA with a HRP/TMB readout (R&D Systems, Minneapolis, MN). The tissue culture supernatants were diluted 50-fold for sTNFR2 detection, and 2.5-fold for sTNFR1 detection. The human serum was diluted 20-fold for both sTNFR2 and sTNFR1 detection. No dilutions were necessary for TNFα detection in either tissue culture supernatants, or human serum. Manufacturers instructions were followed. The optical density was then determined at 450 nm by a spectrometer (Spectramax 190) and values compared to a standard curve to determine unknown concentration.

2.7 Isolation of CD4+ T Cells from Blood

All CD4+ cells were isolated using Dynal magnetic beads methods (Product Nos 113–33D, Invitrogen). Blood and beads were both washed and suspended in Hanks Balanced Salt Solution (HBSS) supplemented with 2% fetal bovine serum. 16µl of fresh blood was added to a 50ml conical tube. The blood in the conical flasks was washed twice. 600µl of beads were used for 48µl of fresh blood (100µl beads per 8µl blood). 200µl beads were added to each 15ml conical tube. The beads in the conical tubes were washed twice with 8ml of washing buffer. During the wash the 15ml conical tubes were placed in a magnet for 1 min and then the supernatant was aspirated. After washing the beads, they were resuspended in 600µl washing buffer per 15ml conical flask. After
being washed, the blood from each 50ml conical flask was added to one set of the 600µl resuspended beads and incubated at 4°C for 20 min with gentle tilting rotation. The 15ml conical tubes containing the blood and beads were then placed in a magnet for 2 min and the supernatant was discarded. The tubes were removed, 8ml of washing buffer was added to each 15ml conical tube and then pipetted up and down vigorously. The suspensions were then added to fresh 15ml conical tubes. This process was repeated 3 times to obtain a high purity of CD4+ cells (97% or more purity). The cell pellets in each 15 ml conical tube were then resuspended in 1ml washing buffer. 80µl of DETACHaBEAD was added to each cell pellet. The cell pellets and DETACHaBEAD were incubated for 1 hour at room temperature with gentle mixing. The tubes were placed in the magnet for 1 minute and the supernatant was transferred to a fresh 15ml conical tube. This step was repeated twice. The cell suspensions from the different 15ml conical tubes were combined in one 15ml conical tube. 10µl of the cell suspension was pipetted onto a hemocytometer and counted.

2.8 DNA Extraction from CD4+ T Cells

5 x 10^6 CD4+ Cells were resuspended in 200µl PBS. The isolation of DNA from these cells was performed with the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. DNA purity was measured using the Spectramax 190.
3.0 Real Time-PCR Quantification of Tregs

Real Time-PCR was performed in white 96-well optical trays with the Roche LightCycler 480 system (Roche Diagnostics, Basel, Switzerland) to a final reaction volume of 20µL, containing 10µL 2-fold Roche TaqMan Probe Master 480, 2µL bisulfite-treated DNA, 1µM of each primer (FOXP3qPCRfw, \textit{GAAATTGTGGGGTG}G \textit{GTATTTGTTTT}; FOXP3qPCRrev, \textit{ATCTACATCTAAACCCCT ATTATCACACCCCC}). The probes (VIC-labeled methylated FOXP3, \textit{TCGGCGTATTC GG}; FAM-labeled unmethylated FOXP3, \textit{AGTTTGGTGTATTTGG}) were added to a final concentration of 166nM. All samples were analyzed in duplicate. After initial denaturation at 95°C for 10 min, the samples were subjected to 40 cycles at 95°C for 15 sec and at 60°C for 1 min [24].

3.1 Statistical Analyses

After data acquisition, the data (flow .fcs files) was imported into Flowjo Software (ver 10.0.7r2 for Mac OS X) for analysis (Flowjo, LLC, 385 Williamson Way, Ashland, CA 97520). A polygate was used to include the lymphocyte cluster, and another more general polygate was used to include all PBMC’s. These polygates were placed in the forward and side scatter dot plots. All samples were double labeled with TmTNFα, TNFR1, or TNFR2 and their respective CD marker.

A series of quadrant gates were used to divide the selected cell population using a two-dimensional dot plot with the x-axis being the CD marker and the y-axis being either TNFR1 or TNFR2. The upper right quadrant provided the percentage of cells, which
were positive for both antibodies. The median fluorescence intensity (MFI) was obtained by gating on the CD positive cells. The Flowjo software was then used to determine the MFI of the cells gated.

The calculated MFI, and upper right percent positive cells values were imported (or manually entered) into Prism Software (Prism 6 (Ver 6.0c) for Max OS X, GraphPad Software, Inc., 7825 Fay Avenue, Suite 230, La Jolla, CA 92037).

The Student’s T test was used to compare differences in means of two groups. In this study it was used to compare variances of percent of cells which were positive for TNF receptor and their CD marker between patients with type I diabetes and controls, as seen in Figure 5. It was also used to compare variances of cytokine production between patients with type I diabetes and controls as seen in Figure 6. The T test was performed as a two-tailed calculation with a 95% confidence interval.

The Spearman correlation was used to analyze and compare age and disease duration to TNFα, sTNFR1, and sTNFR2 production between patients with type I diabetes and control, as seen in Figures 9-11. The Pearson correlation coefficient calculation was also used to determine the significance of spread, as seen in Figures 9-11.
Chapter 3 - RESULTS

3.1 Basal TNFR1 and TNFR2 Expression on Immune Cell Subtype Membranes

The results comparing the expression of TNFR1 and TNFR2 densities on surface membranes of immune cell subtypes between T1D and controls did support our hypothesis. The data shows significant differences between T1D and controls with respect to percent TNFR2 positive CD4, 8, and 14 cells. There was no difference in means or variance of percentage of TNFR1 positive cells between T1D and controls under the conditions tested.

![Patient Percent TNFR2 Positive Cells](image1)
![Control Percent TNFR2 Positive Cells](image2)

![Patient Percent TNFR1 Positive Cells](image3)
![Control Percent TNFR1 Positive Cells](image4)

**Figure 6. TNFR1 and TNFR2 Surface Expression Profiles.** From these dot plots the differences between patients and controls with respect to surface membrane TNFR1 and TNFR2 can be easily discerned. Patients have significantly higher percentages of CD4, 8, and 14 cells with TNFR2. The distribution of TNFR1 across the cell subtypes between patients and controls are the same.
Figure 7. Comparison of TNFR1 Expression Between Patients and Controls. There is no significant difference of expression of TNFR1 on the surface membrane of immune cell subtypes between patients and controls.
Figure 8. Comparison of TNFR2 Expression Between Patients and Controls. Patients and controls show significant differences in the percentage of CD4, CD8, and CD14 that are TNFR2 positive.

3.2 Basal Levels of TNFα, sTNFR1, and sTNFR2 in Serum

The results from comparing serum concentrations of soluble TNFα, sTNFR1, and sTNFR2 between patients with T1D and controls did support our hypothesis (Figure 9).

The data shows significant differences between T1D and controls with respect to all
cytokines and receptors studied. Patients showing complications such as renal failure, retinopathy, and hypertension were placed in a separate group. The patients with complications also showed significant differences in serum concentrations of TNFα, sTNFR1, and sTNFR2 when compared to patients without complications as well as controls. Please refer to Table 2 for a summary of the means and standard errors, and Table 3 for a summary of the P Values for Figure 8.

Figure 9. Basal Serum Levels of TNFα, sTNFR1, sTNFR2. There are significant differences across all subjects and molecules studied. Refer to Table 2 for exact P-values.

Table 2. Summary of the Data Plotted in Figure 8. Listed in this table are the means plus or minus the standard error for the molecules being studied for all study subjects.
Table 3. Summary of P Values Comparing Figure 8 Populations. There are significant differences of basal serum concentration of TNFα, sTNFR1, sTNFR2 between controls, patients, and patients showing complications.

<table>
<thead>
<tr>
<th></th>
<th>Patients compared with Controls</th>
<th>Complications compared with Controls</th>
<th>Complications compared with Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total TNF (pg/mL)</td>
<td>0.0324</td>
<td>&lt;0.0001</td>
<td>0.0014</td>
</tr>
<tr>
<td>sTNFR1 (pg/mL)</td>
<td>0.0177</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>sTNFR2 (pg/mL)</td>
<td>0.0002</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

3.3 Effects of Disease Duration on TNFα, sTNFR1, and sTNFR2 Levels in Serum

Interestingly disease duration shows significant effects with respect to the three molecules being studied. As the duration of T1D increases, patients were shown to have decrease serum TNFα, and increased sTNFR1, and sTNFR2. This effect was only seen when comparing duration of disease; age has no influence in affecting serum concentrations of TNFα, sTNFR1, and sTNFR2 (Figures 10-12 and Tables 4-6).

Figure 10. Effects of Disease Duration on TNFα, sTNFR1, and sTNFR2 Serum Levels. TNF decreases as the duration of T1D increases. Whereas, sTNFR1 and sTNFR2 increase as the duration of T1D increases.
Table 4. Effects of Disease Duration on TNFα, sTNFR1, and sTNFR2 Serum Levels. All three graphs show strong correlations, and all have significant slopes compared to the horizontal.

![Graphs showing correlations](image)

**Figure 11. Effects of Age on TNFα, sTNFR1, sTNFR2 Serum Levels in Patients.** These three graphs show how patient’s age might affect serum TNFα, sTNFR1, and sTNFR2 concentrations.

Table 5. Effects of Age on TNFα, sTNFR1, and sTNFR2 Serum Levels in Patients. Both sTNFR1 and sTNFR2 concentrations are not affect by age. However, an increase in a patient’s age correlates with lower TNFα.

<table>
<thead>
<tr>
<th>Duration of Disease</th>
<th>P Value</th>
<th>Pearson Correlation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total TNF (pg/mL)</td>
<td>0.0004</td>
<td>-0.3414</td>
</tr>
<tr>
<td>sTNFR1 (pg/mL)</td>
<td>&lt;0.0001</td>
<td>0.4022</td>
</tr>
<tr>
<td>sTNFR2 (pg/mL)</td>
<td>0.0083</td>
<td>0.2601</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Age</th>
<th>P Value</th>
<th>Pearson Correlation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total TNF</td>
<td>0.0083</td>
<td>-0.259</td>
</tr>
<tr>
<td>sTNFR1</td>
<td>0.242</td>
<td>0.116</td>
</tr>
<tr>
<td>sTNFR2</td>
<td>0.959</td>
<td>0.005</td>
</tr>
</tbody>
</table>
Figure 12. Effects of Age on TNFα, sTNFR1, sTNFR2 Serum Levels in Controls. These three graphs show how control’s age might affect serum TNFα, sTNFR1, and sTNFR2 concentrations.

Table 6. Effects of Age on TNFα, sTNFR1, sTNFR2 Serum Levels in Controls. Age has no significant effect on serum TNFα, sTNFR1, and sTNFR2 concentrations.

<table>
<thead>
<tr>
<th>Age</th>
<th>P Value</th>
<th>Pearson Correlation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total TNF (pg/mL)</td>
<td>0.817</td>
<td>0.025</td>
</tr>
<tr>
<td>sTNFR1 (pg/mL)</td>
<td>0.702</td>
<td>0.041</td>
</tr>
<tr>
<td>sTNFR2 (pg/mL)</td>
<td>0.053</td>
<td>0.208</td>
</tr>
</tbody>
</table>

3.4 TNFR2 Expression on Monocyte Cell Membranes Following Stimulation

The results from comparing the expression of TNFR2 densities on surface membranes of monocytes between T1D and controls following 48 hours stimulation with various compounds did not support our hypothesis. The data shows no significant differences between T1D and controls with respect to TNFR2 MFI or percent CD14-TNFR2 double positive cells. However, Figure 13 does show the effects of the compounds on monocytes. The stimulated samples were normalized with respect to the non-treated samples. There are less double positive (TNFR2, CD14) cells following IL-2
stimulation. Both patient and control samples increase in the amount of cells containing both markers (TNFR2, CD14) when stimulated with LTA, and LTA-GMCSF. TNFR2 MFI showed no significant change in any of the conditions tested (Figure 13).

![Graph showing changes in CD14-TNFR2 cells and TNFR2 MFI](image)

**Figure 13. Changes in CD14-TNFR2 Positive Cells and TNFR2 MFI.** Ratios of treated to untreated samples were calculated and graphed. No treatment was normalized to 1 and all other samples are expressed as fold differences in comparison to no treatment.

### 3.5 TNFα, sTNFR1, and sTNFR2 Concentrations Following Stimulation

The results from comparing tissue culture supernatant concentrations of sTNFα, sTNFR1, and sTNFR2 between patients with T1D and controls did not support our hypothesis. The data shows no significant differences between T1D and controls with respect to all cytokines and receptors studied. All three markers increased significantly following 48 hours stimulation (Figure 14).
Figure 14. *In Vitro* Induction of TNFα, sTNFR1, and sTNFR2. Comparison of concentrations of TNFα, sTNFR1, and sTNFR2 following 48 hours of stimulation. Tissue culture supernatants were collected and analyzed for these markers.
3.6 Effects of BCG Treatment on Monocytes

The results from these experiments did not support the hypothesis. Both the patients and controls responded equally to the 48hour BCG treatment. Even though no differences were noted between patients and controls, these experiments did shed light on the effects of BCG on cell surface markers, and proliferation of CD14 cells.

BCG significantly increases the percentage of CD14-TNFR2 double positive cells, with the receptor density staying the same regardless of stimulation (Figure 15).

Its effect on TNFR1 density on CD14 surface membranes is extremely drastic. Stimulation with BCG causes a significant decrease in trans-membrane TNFR1. Due to this decrease in density of receptors on the cell membrane, the data also shows a slight but not significant decrease in the percentage of TNFR1-CD14 double positive cells (Figure 16).

There is a slight induction of CD14-TNFα double positive cells, but not significant. The density of trans membrane TNFα is not affected by stimulation with BCG (Figure 17).
Figure 15. Effects of BCG on TNFR2 Expression on Monocytes. BCG induces proliferation of CD14-TNFR2 double positive cells, while keeping the density of the TNFR2 receptor constant.
Figure 16. Effects of BCG on TNFR1 Expression in Monocytes. BCG significantly decreases the receptor density of TNFR1 on CD14 cells following 48 hours incubation. It also causes a slight decrease in the percentage of CD14-TNFR1 double positive cells.
Figure 17. Effects of BCG on Trans Membrane TNF-α Expression on Monocytes. BCG does not significantly change either the percentage of CD14-TmTNFα double positive cells, nor the TmTNFα density on those cells.
3.7 Tissue Culture Supernatant Following BCG Treatment

There were no significant differences in cytokine production following 48 hours stimulation with BCG between patients and controls. Both patient and controls secreted equal amounts of the three markers in response to BCG treatment (Figure 18).

![Figure 18](image)

**Figure 18.** sTNFα, sTNFR1, and sTNFR2 Concentrations Following BCG Treatment. These dot plots show a significant induction of all three markers following 48 hours incubation with BCG.
Chapter 4 - DISCUSSION

As the results show, T1D subjects do show elevated levels of the cytokine TNFα and its receptors in comparison to their healthy control counterparts under certain conditions. Stimulation of PBMCs with TLR-7 agonist IL-2, bacterial TLR-2 agonist LTA, a combination of LTA and GMCSF, and with mycobacterium BCG showed no significant variation between patients with T1D and controls. However, basal serum concentrations and baseline expression of TNF receptors on certain immune cell subtype populations do show differences between people with T1D and controls. Grouping the data by disease duration revealed long-term influences of disease with levels of cytokines. The data shows a longer disease duration is associated with decreased levels of TNFα and an increase in both sTNFR1 and sTNFR2. This supports the hypothesis that TNFα plays a central role in autoimmune disease progression.

Many autoimmune diseases are associated with low TNF levels, and disease progression is clearly demonstrated with anti TNF therapies (Table 7). There is supporting evidence from other studies that anti TNF therapies can be beneficial under certain conditions, treating rather than exacerbating specific types of autoimmune diseases. A perfect example is multiple sclerosis (MS), where patients given anti TNF therapies showed worsening symptoms and progression of the disease. Two patients with MS were enrolled in an early phase I safety trial, one received an anti TNF therapy while the other patient received the placebo. The results from this study showed an increase in demyelinating lesions in the central nervous system (CNS) and decrease immune system activity in the cerebrospinal fluid (CSF) [34]. Another example of this same effect of anti
TNF therapies for patients with MS can be seen in a double blind phase II safety trial. The trial showed that 168 patients with MS received no benefit from the anti-TNF therapy, and also showed worsening of the disease. The rate of worsening was 50% greater in the treated group than from the placebo group [35]. Development of MS and other new onset demyelinating disease has been found in cases where the anti TNF drug Infliximab was being used to treat Colitis and Crohn’s disease [36,37].

Infliximab therapy in 125 Crohn’s patients results, after 24 months, in a high cumulative incidence (57%) of patients developing antinuclear antibodies, two patients developing drug-induced lupus and one patient developing autoimmune hemolytic anemia [38]. In rheumatoid arthritis, therapy with TNF antagonists in all therapeutic forms is associated with relatively common and detectable autoimmune adverse events, including demyelinating disease, confirmed forms of MS, autoimmune hemolytic anemia, type 1 diabetes, a lupus-like syndrome, and cutaneous lupus rashes. Further, 11%–57% of patients develop new or elevated antinuclear antibodies, usually within 1 year of therapy initiation [38–46]. Approximately 7%–15% of patients develop new antibodies against double-stranded DNA [42, 44].

Recently, a case of type I diabetes was reported in a 7-year old girl undergoing treatment for juvenile rheumatoid arthritis with a TNF antagonist [47]. The induction of new onset autoimmunity or the occasional worsening of autoimmunity is an apparent class effect of anti-TNF therapy and is not unique to any given TNF antagonist.
Table 7. Levels of TNFα, TNFR1, and TNFR2 in Various Diseases. A review of the current literature on various autoimmune and non-autoimmune diseases and the role TNFα and its receptors play in disease onset, progression, or treatment.

<table>
<thead>
<tr>
<th>Disease</th>
<th>TNFα as well as other proinflammatory cytokines were elevated in RA patients. Serum levels of both TNFR1 and TNFR2 were both elevated in the serum, with TNFR2 being higher. Levels of these markers were 4-5 fold higher in synovial fluid, suggesting local production in the joint. Neutralization of TNF-α with either monoclonal antibodies or soluble receptors, although not curative, has significant clinical benefit in RA patients.</th>
<th>TNFR1</th>
<th>TNFR2</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rheumatoid Arthritis</td>
<td>Although C Reactive Protein is found significantly lowered in Lupus than RA, all three markers TNFα, TNFR1, and TNFR2 were all found in higher concentrations in Lupus than RA. Several studies have shown anti-TNFα therapies to be beneficial in treating RA, but can inadvertently cause systemic lupus syndrome. Both anti-TNF drugs Infliximab and Entanercept have been implicated in causing drug induced lupus.</td>
<td>-</td>
<td>-</td>
<td>52-56</td>
</tr>
<tr>
<td>Crohns Disease</td>
<td>Levels of TNFα are significantly elevated in patients with Crohns Disease. Successful treatment of disease has been achieved with anti-TNF therapies.</td>
<td>-</td>
<td>-</td>
<td>57,58</td>
</tr>
<tr>
<td>Type I Diabetes</td>
<td>TNFα plays a dual role in type I diabetes pathogenesis depending on the time of induction. Early exposure to TNF seems to exacerbate the progression of disease while late exposure seems to halt the progression. Several studies have shown TNFR1 and TNFR2 elevations to be highly associated with duration of disease, and development of renal failure. Both TNFR1 and TNFR2 are significantly elevated in people with type I diabetes, and even more so when the patients also have renal failure.</td>
<td>-</td>
<td>Serum from patients with MS show significantly elevated levels of sTNFR2.</td>
<td>12,29,59-61</td>
</tr>
<tr>
<td>Multiple Sclerosis</td>
<td>Anti-TNF therapies treating for Juvenile Chronic Arthritis have been shown to precipitate MS. Studies also show an increase in endogenous IFNγ and TNFα up to 2 weeks prior to the development of MS symptoms.</td>
<td>-</td>
<td>-</td>
<td>64-67</td>
</tr>
<tr>
<td>Type II Diabetes</td>
<td>TNFα has been implicated as a causative factor in obesity-associated insulin resistance and the pathogenesis of type 2 diabetes. Neutralization of TNFα is thought to improve insulin resistance. Both TNFR1 and TNFR2 are shown to be elevated in people with T2D who show signs of renal failure. But neither can be used as a marker for insulin resistance. Both TNFα and TNFR2 are elevated in adipose tissue and might be implicated in the increase in insulin resistance. However, measurement and use of sTNFR2 does not seem to be a reliable tool for the identification of obese individuals who are insulin resistant.</td>
<td>-</td>
<td>-</td>
<td>68-73</td>
</tr>
</tbody>
</table>

Future directions for this study might include, determining effects of BCG on specific isolated immune cell subtypes. The bacterial antigen LTA and GMCSF may be switched for different ligands or even antibodies which elicit a stronger response. It would be interesting to observe weather the BCG bacteria can be detected in humans after years of inoculation. Since the goal is to elicit long-term responses from few doses of BCG, then viral vectors might be an option. The use of a bacteriophage would allow
the incorporation of bacterial DNA into the host. With the DNA incorporated into the host, the bacteria’s effect will last a lifetime. The study of BCG’s effects on specific immune cell subtypes also requires the study of their cytokine secretion profiles. It would be interesting to note whether the administration of BCG changes the cytokine secretion profile of certain immune cell subpopulations.

Current literature lacks information regarding BCG’s functional effects, and subsequent contribution it might have towards the protection and possibly cure of T1D. This study represents a step toward defining the use of BCG as a means to an end in type 1 diabetes and possibly other autoimmune diseases, providing further insight into their mechanism of pathogenesis and opportunities for treatment.
REFERENCES


41. Anonymous. Update on the TNF-alpha blocking agents. 2003;


74. Wascher, Thomas C., Jan H. N. Lindeman, Harald Sourij, Teake Kooistra, Giovanni Pacini, and Michael Roden. “Chronic TNF-α Neutralization Does Not Improve Insulin Resistance or Endothelial Function in ‘Healthy’ Men with Metabolic
CURRICULUM VITAE

GEORGES TAHHAN
Address: 1655 Bay Drive Miami Beach, FL 33141
Email: georgestahhan@gmail.com
Phone: (786) 282-4240
Year Born: 1990

Education

<table>
<thead>
<tr>
<th>University</th>
<th>Degree</th>
<th>Start</th>
<th>End</th>
</tr>
</thead>
<tbody>
<tr>
<td>Boston University</td>
<td>Masters of Science: Medical Science</td>
<td>August/2013 – Present</td>
<td></td>
</tr>
<tr>
<td>Harvard University</td>
<td>Course in Neurobiology</td>
<td>August/2012 – December/2012</td>
<td></td>
</tr>
<tr>
<td>University of Florida</td>
<td>Bachelors of Science: Biology</td>
<td>August/2008 – August/2012</td>
<td>Summa Cum Laude</td>
</tr>
<tr>
<td>Miami Beach Senior High School</td>
<td>Summa Cum Laude</td>
<td>August/2004 – June/2008</td>
<td></td>
</tr>
</tbody>
</table>

Research

Graduate Research Fellow Dr. Faustman’s Lab at MGH: July/2014 – May/2015
My master’s thesis project will be focused on the labs phase II clinical trials using BCG vaccinations to stimulate Tumor Necrosis Factor. Endogenous TNF will stimulate the innate immune response stimulating beneficiary T reg cells and increasing apoptosis of disease causing insulin auto-reactive T cells. Dr. Faustman reversed type I diabetes in the non-obese diabetic mouse. I am excited to begin working on the human level, and hopefully provide relief to people suffering from type I diabetes.

Post-Bacc Research Fellow Dr. Notkins’ Lab at NIH: October/2012 – August/2013
My research in Dr. Notkins’ lab is focused on type I diabetes. In specific, discovering the roles of microRNAs embedded in the genes of autoantigens IA-2 and IA-2B. We are also focusing our attention to the regulation and cause of proliferation of polyreactive antibodies. Recently I have started a collaboration between my lab at NIH and my previous lab at the University of Florida focusing on the microRNA expression levels of healthy individuals and people with type 1 diabetes.
Undergraduate Researcher Dr. Atkinson’s Lab at UF: January/2012 - August/2012

My main project, which I wrote my senior thesis on, was single nucleotide polymorphism (SNP) genotyping in conjunction with its functional whole blood assays. The purpose of these two experiments was to stimulate whole blood with antigens, and compare differences in cytokine production between people with different SNP alleles. We also genotyped the blood samples for Haptoglobin-Hemaglobin alleles and compared the genotypes with cytokine production.

Thesis – Masters of Science at BU School of Medicine: May/2015
Control And Induction Of Tumor Necrosis Factor and its Receptors on Human Lymphocytes: A Critical Structure For Immune Regulation

Thesis – Bachelors of Science at UF College of Liberal Arts & Science: August/2012
Disease-Associated SNP rs5979785 Modifies TLR7/8 Responses in Human Peripheral Blood

Oral Presentation – Mid Atlantic Diabetes Symposium: October/2013
Major T1D Auto-antigens: IA-2, IA-2β and GAD65 are Regulated by microRNAs Within the Same Cluster

Poster – Boston University Graduate Research Symposium: March/2015
Bacillus Calmette-Guerin Modifies Expression of Tumor Necrosis Factor Alpha and its Receptors

Poster – NIH Research Symposium: November/2013
Major T1D Auto-antigens: IA-2, IA-2β and GAD65 are Regulated by microRNAs Within the Same Cluster

Poster – Undergraduate Research Symposium - Microbiology: April/2012
Disease-associated SNP rs5979785 modifies TLR7/8 responses in human peripheral blood.

Poster – Undergraduate Research Symposium – Genetics: April/2011
Single nucleotide polymorphism in the F5 gene causing Hemophilia B.

Publication Co-Author Journal Antibodies: February/2015
TNFR1 and TNFR2 Expression and Induction on Human Treg Cells from Type 1 Diabetic Subjects

Publication – Manuscript in preparation:
MicroRNAs Within the 14q32 Cluster Are Involved in the Regulation of the Major Type 1 Diabetes Autoantigens IA-2, IA-2beta and GAD65

Publication – Manuscript in preparation:
The Type I Diabetes Protective TLR8 SNP rs5979785 Associated With Reduced Production of Inflammatory Cytokines.
Experience

Graduate Physiology Tutor at BU School of Medicine: August/2014 – January/2015
I tutored students in the graduate dental physiology course at Boston University School of Dental Medicine. We met once a week to review key concepts learned in lecture, answer any questions regarding the material, and provided any assistance possible to help them succeed in the class.

Academic Tutor/Squash Coach at Squashbusters: February/2014 – August/2014
Squashbusters is an afterschool program for middle and high school students from the Boston area. The students receive academic tutoring for an hour and a half. Academic tutors myself included help the students with any problems they are having in school whether it be preparing for an upcoming exam or homework. Following the completion of their homework they have an hour and a half of squash court time where they practice their form, do drills, and play against each other. This program does wonders for these kids. It instills discipline, and keeps them out of trouble. By the time they complete the program and are applying to college they would have better grades, community service, and have competed in several competitive squash tournaments making them a better-rounded college applicant.

Founder & President of NIH/Walter Reed Racquetball Club: March/2013 – Present
I created the Racquetball Club for staff at both the National Institutes of Health and Walter Reed Army Medical Hospital, regardless of skill level. The club offers a full range of opportunities for both competitive and recreational racquetball players. Each member has the benefits of improving their mechanics, free clinics and the opportunity to compete on the men or women’s intercollegiate teams. I am very proud to say that the club has been received extremely well and is growing every week.

Teaching Assistant for Microbiology Lab at UF: May/2011 - January/2012
After excelling in microbiology lab I was granted the opportunity to become a Teaching Assistant, which I did for two semesters. This taught me essential leadership, time management, and organizational skills.

Collegiate Health Service Corps (CHSC) Member: January/2011 - May/2011
The CHSC organization is focused on serving rural areas in any capacity. My group was focused on informing the citizens of rural areas, the hazards of tobacco use and options they have for cessation of smoking. I also spent time between two clinics shadowing rural family care physicians.

Physician Shadowing VA/Shands Hospital: February/2010 - August/2012
Over the past two years I have shadowed physicians in Critical Care, Emergency, Family Practice, Trauma and Thoracic & Cardiovascular Surgery, and it is through this where I have found my determined interest in surgery.
Volunteer Shands Hospital: January/2010 - May/2010

As a volunteer in the shock trauma and emergency department I was able to gain valuable experience as to what it takes to be an emergency medicine physician, while simultaneously helping the staff in any capacity. I was also part of the PALS program, where I would keep the patients company during their stay in the ER.

Pharmacy Technician Shands Hospital: January/2009 - August/2012

Working as a pharmacy technician exposed me to all the medical services the pharmacy served: CICU, SICU, Surgery, NICU, Pediatrics, Med/Surge, BurnICU, NeuroICU, OBGYN, Trauma, PICU. This job also allowed me to network throughout these services and explore possible career options.

Honors & Awards

Post Bacc Intramural Research Training Award NIH: October/2012

This award provided the opportunity to spend my gap year prior to applying to medical school performing full-time research in Dr. Notkins lab in the National Institute of Dental and Craniofacial Research Experimental Medicine Branch.

Summa Cum Laude from the University of Florida: August/2012

After writing my senior thesis based off of the type 1 diabetes research conducted in Dr. Mark Atkinson’s Lab, the University of Florida granted me the honor of graduating with highest honors from their College of Liberal Arts and Science.

Dean’s List Spring 2012: April/2012

During the spring semester of 2012 I took 18 credits and had a 3.88 GPA. For this reason I was placed on the Dean’s List.

Florida Medallion Scholars Award: September/2008

Due to my high school grades, I was awarded this merit-based scholarship, which allowed me to finish my undergraduate career without debt.

Miami Beach Kiwanis Scholars Award: September/2008

My involvement in the Miami Beach Kiwanis Club allowed me to apply for this scholarship and my above average grades in high school was the factor that granted me this merit-based scholarship of $1000.

Summa Cum Laude from Miami Beach Senior High School: June/2008

I graduated in the top 5% of my high school, which allowed me to graduate with highest honors from the Miami Beach Senior High School.

Extracurricular & Avocation Activities

Active Member of the Society for Advancing Hispanics/Chicanos & Native American in Science (SACNAS)
Former Member of the Racquetball team at the University of Florida
Licensed in-patient pharmacy technician by the state of Florida
Licensed open water scuba diver by National Association of Underwater Instructors (NAUI)

**Language Proficiency**

English – Native: Speaking/Reading/Writing  
Spanish – Native: Speaking/Reading/Writing  
Arabic – Basic: Speaking/Reading/Writing