cholera Toxin B Subunit-Diabetes Autoantigen Fusion Proteins Modulate Dendritic Cell Function and T Cell Morphogenesis

Oludare Jeremiah Odumosu

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Cholera Toxin B Subunit-Diabetes Autoantigen Fusion Proteins Modulate Dendritic Cell Function and T Cell Morphogenesis

by

Oludare Jeremiah Odumosu

A Dissertation submitted in satisfaction of the requirements for the degree of Doctor of Philosophy in Biochemistry

June 2011
Each person whose signature appears below certifies that this dissertation in his/her opinion is adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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Nathan R. Wall, Assistant Professor of Biochemistry and Microbiology
ACKNOWLEDGEMENTS

I thank God for giving me the grace and strength to complete this degree. This achievement took a complete dependence on God and some very important people that he placed in my life. Every successful events in my life has been a result of the collective efforts of several people and in some cases, their efforts surpassed my own endeavors. The first person I would like to thank is my mentor, William Langridge, who allowed me to be scientifically creative in a very nurturing environment. He believed in me and allowed me to flourish in his lab. Dr. Langridge, we took a seemingly impossible thought of pursuing dendritic cells as targets of suppression of Type 1 diabetes and successfully made it the main focus of our lab in four years. Thank you for encouraging me to go for it the day I walked in your office with a scratch paper of ideas. Here we are now! Thank you for the countless number of hours over the last four years brainstorming ideas with me, editing manuscripts, spending late nights tweaking presentations and giving me both scientific and fatherly advice. I also like to acknowledge the patience and dedication of my Ph.D. committee members—Kimberly Payne, Penny Duerksen-Hughes, Alan Escher, Daila Gridley, and Nathan Wall. Their scientific wisdom, guidance and friendly advice brought me safely to this point. In particular, Dr. Payne is the reason why this entire project took off in the first place. I walked into her office with a crazy idea about differentiating monocytes from cord blood from the hospital and she immediately found a way to work me into her protocol.

I am eternally grateful to so many of the scientists, students, and especially to the office staff of the graduate school for their laboratory assistance, expertise, counsel and friendship. The following list is by no means exhaustive but I am especially grateful to
the following people for being a positive part of my graduate school experience: My lab mates—Dequina Nicholas (my little sister), and Hiroshi Yano; My colleagues and research associates—Ineavely Baez, Abby Weldon, Terry Milford, Abigail Benitez, Gay Asumen, Jessica Slater, Jonathan Aspen. Dr Salma Khan, I thank you very much for your sisterly and scientific advice. The front office staff at the Center for Health Disparities and Molecular Medicine—Lori Hoewing, Ann Bradshaw, Debra Rosenstock are wonderful women. They were God sent to me. They played the role of mother to me more than they even knew. Marissa Fulache, Nancy Bethel, Cindy Messer and Jacqueline Brower were particularly helpful in navigating the system here at LLU Basic Sciences. Thank you very much for you guidance and friendship. Drs. Istvan Fodor, Nathan Wall, Bela Dénes were particularly helpful in directing my experiments and providing guidance. I would like to thank Rafael Canizales, the assistant dean for administrative affairs, for his editorial assistance in preparing the final draft of my dissertation. My deepest apologies if I have left out anyone.

Dr. Stephen Matheson, my undergraduate research mentor, I am most grateful to you. I share this achievement with you because you found this scared and anxious new international student in the hallway on my first day in the biology department at Calvin College in 2001. That day, you saw something in me that I didn’t yet see in myself. You took me under your wings as your first research student at Calvin College and trained me to be a fine creative scientist. Four summers in the Art Albert signal transduction lab at the Van Andel research Institute to building our own lab in the Biology department taught me how to manage a lab and still be scientifically productive and efficient. I owe where I am today to your guidance, your teaching and outstanding scientific research
techniques, confidence and encouragement. I hope I made you proud.

I would like to thank several family members who deserve to share this achievement. Foremost, I would like to thank my parents Olusayo and Olutayo Odumosu. Your support for me knows no limit, always looking out for my best interests. You raised four children, of which I am your oldest child, but you sure came through for us every time we needed you and still will do everything and anything for us. You are both my parents and my friends. I want you to know how special you are to me—I think of you as my nurturing, protective, dear friends full of wisdom and life. Thank you for your great and uncountable sacrifices. I live to make you proud will always love you.

My siblings Tosin and Femi deserve great credit for shaping my life. I admire the tenacity with which you face your struggles daily. I hope that I set a decent example as your older brother. Above all, both of you are constant streams of inspiration and love for me. I love you. To my youngest brother Dayo, we have well over 9 years between us and I missed most of your growing years. I thank you because in your quiet way, you show you that you love me. Thank you for the many nights you spent with me in the lab on your vacation as I struggled to clone a difficult gene product. I love you and pray that you grow up to do greater things than I have done.

To all my extended family and loyal friends, you have done more than I could ever express to keep me sane and feeling loved. It has been wonderful to feel the love and support of blood relatives and love relatives. I thank you for your support, encouragement, and great memories. The Ojelabi family: uncle Tim, aunty Christiana, Anthony (junior), Alex and John. Thank you for being my guardians and source of sustenance. On the weekends, aunty would always send me back with a car full of food
to feed a hostel! I pray that you sons will make you proud. Pops and Mama Brink—you are more than just my friend’s parents; you took me as one of your own. Thank you for your love and support. I often looked forward to Sunday lunches at the Brinks. Thank you all member of Rosewood Church in Bellflower, I felt your prayers every step of the way. Biola and Hannah, thank you for coming to visit me and sitting through some of my classes. Sis. Bimbo, Bolade (my wonderful cousins), Nathan and Gihane Jeremie-Brink (brother and sis from other mothers!), Jermale and Anissa Adkins and little Malachi (Grand Rapids family!), Becky Vanderzee (the back cave is the best escape in the world!), Dorian Stith, Sica Lyles, Drs. Yemi and Kemi Adeoye and my God daughter Tamilore (you make me think I can be good father), Maximo Carmona, Jide Faseyi, Joel Howerlda, and Carl Sankey. You all are such a joy to me. Your support, encouragement, prayers, and love carried me through. The Umeh family, The Fakoredes, my many uncles and aunties in Nigeria, Canada, the US and the UK, my chaplain and big sister-Dilys Brooks, and Olaide Ajayi, Wow! You all played very special roles in my life that I cannot ever repay you for. Thank you all for your immeasurable support through this journey. We made it!

I will not be here today without the support of Dr Harvey and Phillys Bratt (My dearest adopted grandparents). You mentored me during my undergraduate studies in Grand Rapids and remained committed to my success in life even after I moved to California. I am so blessed that you call me you grandson and love me as your own. The mentoring scholarship that brought us together definitely changed my life for the better. I commend Calvin College for the initiative to pair students with mentors in the community. My case is clearly a success story. As the reality of aging reminds us that
our time together is only by grace, I am where I am today because you came and found me in my dorm room that one afternoon. I love you very much.

I want to especially thank my dear friend and confidant Jasmine Nichole Swenson. If you have a friend like her, you would be able to face any storm in life with confidence. Jasmine, since I met you in 2006, you showed me daily what it is to be a true friend who is willing to do anything for a friend. Sometimes I wondered why you believed in me so much. You are my adorable, playful, spiritually minded, big-hearted, irresistibly elegant and extraordinarily lovely friend who endured all my antics, faults and foibles. You were here when I missed my family, when I needed a stern word, when I needed a shoulder to cry on or someone to laugh with. You stood by me, braved many storms with me and pulled me through the darkest tunnels of my graduate school experience, some experiences I couldn’t even share with family and friends. You often revived my experiment-weary soul with your humor and tenderness despite my tendency to wall myself off whenever I was down. With you I pondered my belief in virtue and the importance of integrity and loyalty. I questioned how rational thought can edify or possible destroy the essence of a person. Jas you helped me understand that sometimes there is great triumph in surrendering to the irrationality of life and the power of its inevitability. I love you so much and remain eternally indebted to you.

Oludare Odumosu a.k.a Dare
DEDICATION

This doctoral dissertation is dedicated to my grandmothers: Dorcas Odumosu (mama-Ife), Deborah Oluokun (mama-ogbomosho) and Yewande Imasogie (Mama-Imasogie). The three of you passed away in the four years it took me to complete this degree. As sad as it is to celebrate this achievement without you, I am confident that you are celebrating this success with me beside my heavenly father. I miss you every day. I would also like to dedicate this work to my parents, who stood by me, supported me, and guided me through life. I love you.
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<th>Description</th>
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<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen-presenting cell</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CDC</td>
<td>Centers for Disease Control</td>
</tr>
<tr>
<td>CFA</td>
<td>Complete Freud’s Adjuvant</td>
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<tr>
<td>C.I.</td>
<td>Confidence interval</td>
</tr>
<tr>
<td>CT</td>
<td>Cholera Toxin</td>
</tr>
<tr>
<td>CTB</td>
<td>Cholera toxin B subunit</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T-lymphocyte</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>Cytotoxic T-lymphocyte antigen-4</td>
</tr>
<tr>
<td>CTX</td>
<td>Cholera toxin (holotoxin)</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>EAE</td>
<td>Experimental autoimmune encephalomyelitis</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>Foxp3</td>
<td>Forkhead box P3</td>
</tr>
<tr>
<td>GAD</td>
<td>Glutamic acid decarboxylase</td>
</tr>
<tr>
<td>GALT</td>
<td>Gut-associated lymphoid tissue</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
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<td>--------------</td>
<td>-------------</td>
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<tr>
<td>HLA</td>
<td>Human leukocyte antigen (i.e. MHC)</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>HSP</td>
<td>Heat shock protein</td>
</tr>
<tr>
<td>HSV</td>
<td>Herpes simplex virus type-1</td>
</tr>
<tr>
<td>IA-2</td>
<td>Insulinoma associated autoantigen-2</td>
</tr>
<tr>
<td>IBD</td>
<td>Inflammatory bowel disease</td>
</tr>
<tr>
<td>ICA</td>
<td>Islet cell autoantigen</td>
</tr>
<tr>
<td>IDDM</td>
<td>Insulin-dependent diabetes mellitus</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>INS</td>
<td>Proinsulin (human)</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl-β-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>LADA</td>
<td>Latent autoimmune diabetes in adults</td>
</tr>
<tr>
<td>LAP</td>
<td>Latency-associated peptide</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LTB</td>
<td>Heat-labile enterotoxin from <em>E. coli</em></td>
</tr>
<tr>
<td>MALT</td>
<td>Mucosa-associated lymphoid tissue</td>
</tr>
<tr>
<td><em>mas</em></td>
<td>Mannopine synthase bidirectional promoter</td>
</tr>
<tr>
<td>MCS</td>
<td>Multiple cloning site</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MOG</td>
<td>Myelin oligodendrocyte glycoprotein</td>
</tr>
<tr>
<td>NOD</td>
<td>Non-obese diabetic mouse</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Term</td>
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<td>--------------</td>
<td>-----------</td>
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<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PBST</td>
<td>Phosphate-buffered saline + Tween-20</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>pNOS</td>
<td>Nopaline synthase gene promoter (<em>A. tumefaciens</em>)</td>
</tr>
<tr>
<td>PSA</td>
<td>Polysaccharide A</td>
</tr>
<tr>
<td>RA</td>
<td>Rheumatoid arthritis</td>
</tr>
<tr>
<td>RPM</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>R/T</td>
<td>Room temperature</td>
</tr>
<tr>
<td>RTB</td>
<td>Ricin toxin B chain</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SLE</td>
<td>Systemic lupus erythematosus</td>
</tr>
<tr>
<td>STB</td>
<td>Shiga-toxin B subunit</td>
</tr>
<tr>
<td>T1D</td>
<td>Type 1 diabetes</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-buffered saline + Tween-20</td>
</tr>
<tr>
<td>TCR</td>
<td>T-cell receptor</td>
</tr>
<tr>
<td>T&lt;sub&gt;eff&lt;/sub&gt;</td>
<td>T effector cells (i.e. Th1, Th2, Th9, Th17, and Tfh)</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>Th</td>
<td>T helper cell</td>
</tr>
<tr>
<td>Tfh</td>
<td>T follicular helper cell</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptors</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>iT&lt;sub&gt;regs&lt;/sub&gt;</td>
<td>Inducible T-regulatory cells (i.e. Th3 and Tr1)</td>
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<td>nT&lt;sub&gt;reg&lt;/sub&gt;</td>
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</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
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Type 1 diabetes mellitus is a chronic inflammatory disease in which the insulin
and glutamic acid decarboxylase producing β-cells of the pancreatic islets are killed by
autoreactive cells of the immune system in response to a loss of tolerance. Early
experiments showed that self-tolerance could be partially restored by oral inoculation
with small amounts of insulin. Later experiments showed that insulin autoantigen induced
immune tolerance could be greatly enhanced by linkage of β-cell autoantigens (Auto Ag)
to adjuvant molecules such as the non-toxic cholera enterotoxin B subunit (CTB).

To identify mechanisms responsible for CTB-autoantigen fusion protein
suppression of diabetes autoimmunity, dendritic cells (DCs), a major class of antigen
presenting cells (APC) involved in restoration of immunological tolerance, were
differentiated from umbilical cord blood monocytes and incubated with recombinant
CTB- pro-insulin and CTB-glutamic acid decarboxylase adjuvant-autoantigen fusion
proteins. The fusion proteins were shown to dramatically suppress DC biosynthesis of
costimulatory factors and proinflammatory cytokines IL-12 and IL-6 required for
autoreactive T cell development. In addition, a substantial increase in DC secretion of the
anti-inflammatory cytokine IL-10 was also detected further confirming the previous
observation. Naïve T cells incubated with CTB-autoantigen inoculated DCs developed into mature T cells that synthesized IL-10 and TGF-β cytokines indicative of Th2, Tr1 and Th3 regulatory T cell populations involved in suppression of CD4+ and CD8+ T lymphocytes responsible for the initiation of diabetes autoimmunity. Further, vaccine inoculated DCs suppressed T cell pro-inflammatory cytokines IFN-γ, IL-17 and IL-2 guiding T cell morphogenesis in the direction of tolerance rather than toward inflammation responsible for diabetes onset. In summary: the experimental results presented in this Dissertation indicate that ex vivo vaccination of human DCs favors immunological suppression of diabetes development and suggests that mechanisms of immunological tolerance responsible for multicomponent vaccine suppression of diabetes onset and possibly progression are comparable in humans and in animals.
CHAPTER ONE
INTRODUCTION

Type 1 Diabetes
Diabetes Etiology

Juvenile onset autoimmune diabetes also known as type 1 diabetes (T1D) or insulin-dependent diabetes mellitus (IDDM) is considered to be the most serious metabolic disease of children and adolescents. High blood sugar (hyperglycemia) and inflammation of the pancreatic islets (insulitis) result from a breakdown in immunological homeostasis leading to loss of immunological tolerance. Consequently, the pancreatic islets of Langerhans insulin-producing \( \beta \)-cells are destroyed by the body’s immune system gone rogue. Chronic insulitis and progressive \( \beta \)-cell death are initiated by islet autoantigen stimulated maturation of antigen-presenting cells, largely dendritic cells (DC) part of the host innate arm of the immune system. Autoantigen activated DCs migrate to pancreatic and other peripheral lymph nodes where they induce naïve T lymphocyte (Th0) development into autoreactive CD4+ T helper cell and CD8+ cytotoxic T lymphocytes, as well as B cells that synthesize autoreactive antibodies (Atkinson, Bowman et al. 1994; Atkinson and Maclaren 1994; Tisch and McDevitt 1996; Han, Serra et al. 2005). The progressive loss of \( \beta \)-cell function causes increased insulin deficiency eventually resulting in the onset and progression of hyperglycemia, (frank diabetes) (**Fig 1.1**).

Young and older T1D patients alike must monitor their blood sugar levels and
inject insulin several times a day for the remainder of their lives or risk diabetic shock, coma and death. While daily multiple insulin injections suppress diabetes symptoms, this therapy does not provide consistent physiological levels of insulin. Without insulin, there is a problem of chronic glucose dysregulation resulting in unacceptable levels of disability and mortality. Symptoms of hyperglycemia include, but are not limited to, frequent thirst and urination as the kidneys attempt to cope with efforts at eliminating excess sugar from the bloodstream. In addition, extreme hunger, weight loss, fatigue, and blurred vision may occur due to cellular glucose deprivation. Long-term complications include a progressive risk for neural and circulatory failure resulting in amputation of the extremities, blindness, kidney failure, heart attack and stroke reducing average life expectancy by an average of 15 - 20 years (Libby, Nathan et al. 2005). See Table 1.1.
Table 1.1. Secondary Effects of Diabetes Onset from Diabetes Health Statistics (U.S.A.)

<table>
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<th>Condition</th>
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| Heart disease and stroke   | - Heart disease and stroke account for about 65 percent of deaths in people with diabetes.  
- Adults with diabetes have heart disease and stroke death rates about 2 to 4 times higher than adults without diabetes. |
| High Blood Pressure        | - About 75 percent of adults with diabetes have blood pressure greater than or equal to 130/80 mm Hg or use prescription medications for suppressing hypertension.                                                   |
| Blindness                  | - Diabetes is the leading cause of new cases of blindness among adults aged 20 to 74 years.  
- Diabetic retinopathy causes 12,000 to 24,000 new cases of blindness each year.                                                                 |
| Kidney Disease             | - Diabetes is the leading cause of kidney failure, accounting for 44 percent of new cases in 2005.  
- In 2005, 46,739 people with diabetes began treatment for end-stage kidney disease in the United States and Puerto Rico while 178,689 people had end-stage kidney disease that required chronic dialysis or kidney transplantation. |
| Nervous System Disease     | - About 60 to 70 percent of people with diabetes have mild to severe forms of nervous system damage. The results of such damage include impaired sensation or pain in the feet or hands, slowed digestion of food in the stomach, carpal tunnel syndrome, erectile dysfunction, or other neuropathological problems.  
- Almost 30 percent of people with diabetes ages 40 years or older have impaired sensation in the feet—for example, at least one area that lacks feeling.  
- Severe forms of diabetic nerve disease are a major contributing cause of lower-extremity amputations. |
| Amputations                | - More than 60 percent of non-traumatic lower-limb amputations occur among people with diabetes.  
- In 2004, about 71,000 non-traumatic lower-limb amputations were performed in people with diabetes. |
| Dental Disease             | - Periodontal or degenerative gum, disease is more common in people with diabetes. Among young adults, those with diabetes have about twice the risk as those without diabetes.  
- People with poorly controlled diabetes—A1C greater than 9 percent—were nearly three times more likely to have severe periodontitis than those without diabetes.  
- Almost one-third of people with diabetes have severe periodontal disease with loss of attachment of the gums to the teeth measuring 5 millimeters or more. |
Table 1.1. Continued.

| Complications of Pregnancy | • Poorly controlled diabetes before conception and during the first trimester of pregnancy among women with T1D can cause major birth defects in 5 to 10 percent of pregnancies and spontaneous abortions in 15 to 20 percent of pregnancies.  
• Poorly controlled diabetes during the second and third trimesters of pregnancy can result in excessively large babies, posing a risk to both mother and child during delivery. |
| Other Complications | • Uncontrolled diabetes often leads to biochemical imbalances that can cause acute life-threatening events, such as diabetic ketoacidosis and hyperosmolar, or nonketotic, coma.  
• People with diabetes are more susceptible to many other illnesses and, once they acquire these illnesses, often have worse prognoses. For example, they are more likely to die with pneumonia or influenza than people who do not have diabetes.  
• People with diabetes ages 60 years or older are two to three times more likely to report an inability to walk a quarter of a mile, climb stairs, do housework, or use a mobility aid compared with people without diabetes in the same age group. |
| Health Disparities | • Non-Hispanic white youth had the highest rate of new cases of type 1 diabetes—24.8 per 100,000 per year among those younger than 10 years and 22.6 per 100,000 per year among those ages 10–19 years (Source: SEARCH for Diabetes in Youth Study 2002-2005) |

Figure 1.1. Basic Mechanism of Type 1 Diabetes Inflammation and Vaccine Action

Antigen-presenting cells, more specifically dendritic cells, process endogenous pancreatic beta cell autoantigens (Insulin, GAD…) in lysosomal vesicles and transfer peptide fragments of the autoantigen to MHCII molecules that migrate to the plasma membrane and present the autoantigen fragments to cognate T cell receptors on naïve T helper cells (Th0), left portion of Figure 1.1. Dendritic cell processing of the autoantigen stimulates biosynthesis and secretion of the inflammatory cytokine interleukin 12 (IL-12) which stimulates naïve Th0 lymphocytes to undergo morphogenesis into autoreactive effector T helper cells (Th1). The autoreactive Th1 cells secrete inflammatory cytokines such as IFN-gamma and IL-2 that stimulate cytotoxic lymphocytes (CTL) to secrete nitric acid, peroxide and several additional inflammatory cytokines such as TNF-α and IL-1β that stimulate pancreatic islet inflammation (insulitis). Chronic insulitis results in progressive pancreatic beta cell death resulting in an increasing insulin deficiency and a progressive increase in blood sugar (hyperglycemia). Oral delivery of small amounts of islet autoantigens (right portion of panel); exert a specific protective antigen therapeutic effect by stimulating dendritic cell secretion of the anti-inflammatory cytokine IL-10. Secretion of IL-10 stimulates naïve cognate naïve Th0 lymphocytes to undergo morphogenesis into anti-inflammatory CD4+ Th2 helper cells that in turn secrete IL-10, which suppresses autoreactive Th1 cell development and decreases the potential for insulitis onset. Alternatively, naïve Th0 cells may develop into one of several subclasses of regulatory T cells (Treg), which can block dendritic cell activation, Th2, Th1 and CTL development leading to prevention of insulitis, continued insulin secretion and maintenance of immunological homeostasis (Ploix, Bergerot et al. 1999; Liu, Iyoda et al. 2002).
Epidemiology of Type 1 Diabetes

According to the National Institutes of Diabetes and Digestive and Kidney Diseases-NIDDK, the current incidence rate of T1D in the U.S is approximately 19.7 per 100,000 annually. Among youth ages 10 years or older, the incidence rate is 18.6 per 100,000 children (National Diabetes Statistics Fact Sheet, 2010). Stratified by race and ethnicity, non-Hispanic white youth had the highest rate of new cases of T1D with 24.8 cases per 100,000 per year among those younger than 10 years and 22.6 cases per 100,000 per year among those of ages 10–19 years (National Diabetes Statistics Fact Sheet, 2010). Worldwide, it is estimated that there are 65,000 newly diagnosed cases of T1D per year for children less than 15 years of age (source: International Diabetes Federation, 2009). Over the past 50 years, T1D prevalence has experienced an exponential rise, which seemingly has more to do with environmental factors than genetic causation (Gillespie, Bain et al. 2004). Worldwide, the incidence of T1D in children under 14 yr has increased on all continents except Central America and the West Indies from 1990 – 1999 (2006). In this study of 43,013 cases of T1D out of a population size of 84 million (representing 114 populations in 57 countries), the average rise in incidence of T1D from 1990 to 1994 was 2.4%. From 1995 to 1999 the worldwide incidence rose to 3.4%. Figure 1.2 shows the worldwide incidence of Type 1 diabetes in children ≤14 years of age.

In a large longitudinal study conducted in 17 European countries and based on current T1D statistics, it is predicted that between the years 2005 and 2020, new cases of T1D in European children younger than five years of age will double, while the prevalence of T1D is expected to rise by 70% to 160,000 cases in children under fifteen
Figure 1.2: Worldwide incidence of Type 1 diabetes in children ≤14 years of age. Values presented are age-adjusted and listed in descending order of incidence (per 100,000 people per year). Note that many of the countries with the highest incidence of diabetes are located north or south of the equatorial belt, where sunshine is less intense annually and therefore dermal vitamin D synthesis is lower, on average, suggesting a role for vitamin D in diabetes onset. Other countries with a high incidence of people with T1D are industrialized nations, where children and adults spend more time indoors and out of the sun. Despite being in a sunny location, apparent anomalies like Kuwait—the country with the 4th highest incidence of T1D—might have increased numbers of people with autoimmune diabetes due to their darker skin color and a tendency to clad themselves extensively in clothes that may block vitamin D synthesis.
years (Patterson, Dahlquist et al. 2009). The disparity between T1D prevalence and incidence across the globe may be explained by a multi-factorial and complex combination of factors involving population genetics, socioeconomic status, exposure to pathogens, geographic location, skin color (melanin density), diet (e.g. vitamin D supplementation, consumption of cow’s milk, nitrates, etc.) and genetics.

The first sign of disease onset in patients with T1D is the presence of autoantibodies produced by self-reactive B-cells. Serological analyses showed that some children develop autoantibodies as early as three months of age (Kukko, Kimpimaki et al. 2005). In T1D patients, the number of autoantibodies to insulin (INS), glutamic acid decarboxylase (GAD), or islet cell autoantigen-512 (ICA512) is indicative of the relative risk for development of diabetes among first-degree relatives (Verge, Gianani et al. 1996; Verge, Gianani et al. 1996). The five-year risk of a diabetes outcome was as follows:

- No positive islet autoantibodies = 0%;
- Presence of autoantibodies to one islet autoantigen = 15%;
- Presence of autoantibodies to two islet autoantigens = 44%;
- Presence of autoantibodies to three islet autoantigens = 100% risk.

Anti-INS antibodies can be found in 50-70% of T1D children (Greenbaum, Palmer et al. 1992) and anti-IA-2 antibodies are seen in 70-80% of cases (Gorus, Goubert et al. 1997). Anti-GAD antibodies occur in 70-75% of T1D patients. However, it was found that 1-2% of the general population generates anti-GAD antibodies yet still retains normal pancreatic function (Sanjeevi, Falorni et al. 1996). This observation suggests that autoreactive lymphocytes may be present in healthy individuals, but may be down regulated by tolerogenic mechanisms after they escape thymic selection early in life.
Disease onset due to hyperglycemia (>250 mg/dl blood glucose), can take years to develop following initial detection of autoantibodies (Eisenbarth 1986). While autoantibodies produced by B-cells are indicative of disease onset, they are not thought to serve a pathogenic role (Yoon and Jun 2005). The main role of B-cells in pancreatic islet beta cell destruction may be related to their ability to present β-cell antigens to autoreactive T-cells, which in turn activates cytotoxic lymphocyte destruction of the β-cells. Hyperglycemia, the major clinical symptom of overt diabetes, occurs only after >80-90% of the insulin-producing β-cells have been destroyed by cytotoxic T-lymphocytes and macrophages (Knip and Siljander 2008). Although β-cell destruction continues throughout the lifetime of the T1D patient, it appears that insulin-producing cells continue to be differentiated to a small degree within the pancreas and can still be detected in >88% of patients with frank diabetes (Meier, Bhushan et al. 2005). This condition suggests that if a targeted immune suppression therapy such as that proposed in this dissertation was to be applied, newly formed β-cells could be protected from autoimmune destruction, resulting in improved possibility for restoration of normoglycemia. Islet transplant or stem cell therapy may supplement and even accelerate the regeneration of islet beta cells, but only if autoreactive lymphocyte activity can be persistently blocked to prevent their destruction of nascent β-cells.

Factors Affecting the Etiology of Type 1 Diabetes

Genetics

Haplotype differences in the major histocompatibility complex (MHC) genes responsible for self-antigen presentation to the immune system may account for many
individuals with increased susceptibility to autoimmunity. Specifically as it applies to TID, disease susceptibility is determined by variations in the HLA-DQ (an αβ heterodimer of the MHC class II cell surface receptor found on antigen presenting cells) on chromosome 6 beta genes, while additional alleles provide significant resistance to T1D development (Todd, Bell et al. 1987). More recently, a genome-wide search identified alleles at 41 loci that increase the risk of T1D development, including variations in several important interleukin (IL) genes and surface markers involved in immune activity or β-cell function such as IL10, IL19, IL20, GLIS3, CD69 and IL27 (Barrett, Clayton et al. 2009).

In some cases, random genetic recombination of T-cell receptor (TCR) genes for T-cell specific antigenic epitope recognition can result in TCRs that bind self-peptides. Normally, autoreactive T-cells are selected and eliminated in the thymus (thymic selection). However, it is possible that some of these autoimmunity-stimulating T-cells may escape this process and become suppressed by regulatory immune cells located in the periphery of healthy individuals. In autoimmune disease victims, the regulatory capacity of the immune system is no longer able to downregulate autoreactive T-cell development. The majority of epitopes responsible for induction of TID autoimmunity are fragments of proinsulin —INS (Palmer, Asplin et al. 1983), glutamic acid decarboxylase—GAD (Baekkeskov, Aanstoot et al. 1990), insulinoma associated autoantigen-2 (1A-2 and 1A-2β/phogrin) (Bonifacio, Lampasona et al. 1995; Lu, Li et al. 1996), carboxypeptidase-H (Castano, Russo et al. 1991), islet cell autoantigen-69—ICA69 (Pietropaolo, Castano et al. 1993), GM gangliosides (Nayak, Omar et al. 1985), imogen-38 (Arden, Roep et al. 1996), sex-determining region Y-related protein-13—
SOX13 (Kasimiotis, Myers et al. 2000). A compilation of the predominant epitopes recognized by CD4 and CD8 T-cells from humans and mice with TID has been published by Di Lorenzo, T.P. et al. (2007). Recently, autoantibodies to zinc transporter protein—ZnT8 were found in 60 – 80% of patients with new onset T1D, including 26% that uniquely had ZnT8 antibodies in the absence of antibodies that recognize the usual markers of β-cell autoimmunity—INS, GAD, IA-2, and ICA (Wenzlau, Juhl et al. 2007). Detection of T1D at disease onset was achieved in 98% of individuals when antibodies were screened against a combination of INS, GAD, IA-1, and ZnT8 proteins.

**Molecular Mimicry**

Since genetics alone only accounts for approximately 50% of diagnosed T1D cases, environmental influences on disease initiation cannot be ignored (Drescher and Tracy 2008). Although poorly developed, there is increasing evidence that exposure to certain pathogens may increase susceptibility to TID. Particularly, molecular mimicry, epitope spreading, or direct bystander activation by pathogen epitopes with similar structure or binding capacity to human proteins may lead to cross-reactive antibody or T-cell receptor formation that can trigger an immune attack against self-tissues (Olson, Croxford et al. 2001). To date, 14 different viruses have been reported that may be strongly associated with T1D in humans and various animal models (Jun and Yoon 2003). The Coxsackie B enterovirus (a member of the picornaviridae) is one of the viruses most suspected of eliciting a cross-reactive immune response (Drescher and Tracy 2008).
Immune Suppression of Diabetes Autoimmunity

Dendritic Cells are Sensors of Infection and Activate T cells in Peripheral Lymphoid Organs

Dendritic cells (antigen presenting cells that bridge the innate and adaptive arms of the immune system); continuously patrol the microenvironment for invading pathogens. DCs are not a single cell type, but a heterogeneous collection of cells that arise from distinct, bone marrow-derived or hematopoietic lineages (Caux, Massacrier et al. 1997; Reid 1997). That is, subsets from different tissues have been shown to possess a differential morphology, phenotype and function (Appendix 5). Three-color flow cytometry revealed two subpopulations of HLA-DR+ DCs, characterized primarily by the phenotypes CD11c- HLA-DR+ (lymphoid appearance) and CD11c+ HLA-DR+ (monocytoid morphology) respectively (Thomas and Lipsky 1994; Robinson, Patterson et al. 1999). Further, both subsets lack expression of the LC marker CD1a and express only low levels of adhesion and co-stimulation molecules CD80, CD86 and CD40, suggesting that these cells are relatively immature (Carnevali, Bonati et al. 1980; O'Doherty, Peng et al. 1994; Robinson, Patterson et al. 1999). However, when cultured, both populations develop into cells with typical DC morphology that express high levels of adhesion and co-stimulatory molecules and possess potent allostimulatory function (O'Doherty, Peng et al. 1994). It has been suggested that the so-called myeloid and lymphoid-derived subsets of DCs perform specific stimulatory or tolerogenic function, respectively. Regardless of their subset, the ability to stimulate naïve T cell proliferation appears to be shared between these various DC subsets (Hart 1997; Shortman and Caux 1997). DCs become activated by inflammatory or pathogenic products derived from microbes, toxins or from damaged tissues (Viney 2001). The acquired stimulus induces DC mobilization from
non-lymphoid tissues to draining lymphoid organs via afferent lymphatic vessels. DCs are thought to exist either in an immature or mature state. The capacity of DCs to induce immunity depends on their state of development frequently referred to as “maturation” (Figure 1.3). During maturation, the DCs convert antigens to immunogens, which they transfer via MHC molecules from intracellular vesicles to the cell surface. In addition during activation, DCs express adhesion and co-stimulatory molecules (e.g. CD86, CD80, CD83 and CD40), for establishment of cell-to-cell interactions and the release of soluble cytokines, chemokines and enzymes responsible for regulating cellular communication and mobilization (Banchereau and Steinman 1998; de Saint-Vis, Fugier-Vivier et al. 1998; Guermonprez, Valladeau et al. 2002; Jiao, Lo-Man et al. 2002).

Immature DCs are characterized by very low levels of synthesis and membrane presentation of surface co-stimulatory molecules and by low levels of IL-6, IL-1β, TNF-α and IL-12 inflammatory cytokine biosynthesis (Hackstein and Thomson 2004). In contrast, DC maturation triggers an increase in the expression of inflammatory cytokines. DCs become terminally matured when they finally make contact with cognate T lymphocytes in the specialized microenvironment of lymph node tissues, which permits intimate physical interaction among DCs and naïve T lymphocytes referred to as the immunologic synapse (Merwe 2002; Gogolak, Rethi et al. 2003). Primarily, DCs function as specialized cellular sensors of pathogenic changes that occur within a tissue. In addition to detection of pathogenic changes, a second major DC function is transmission of this information to cognate T lymphocytes for further processing leading to an amplified immune response (Reis e Sousa 2001). The immunological synapse formed between the DC and the T cell is stabilized by multiple co-stimulatory and adhesive
Figure 1.3. The antigen presentation and T cell activation functions of mature dendritic cells. Differentiated (mature) dendritic cells are professional antigen presenting cells located in tissues throughout the body and especially adjacent to the epidermal mucosae. Mature DCs are able to activate both CD4+ helper and CD8+ cytotoxic T lymphocytes based on the nature of the pathogen proteins taken up and processed by the antigen-presenting cell. Helper T lymphocytes can also support B cell differentiation and antibody production (Gogolak, Rethi et al. 2003)
molecules, while the immunological specificity of the signaling complex is maintained by interactions between the MHC-Peptide–TCR complex (Figure 1.3). The expression of co-stimulatory molecules and cytokine receptors on DCs in combination with the pattern of DC released cytokines are responsible for differentiation of the naïve CD4+ helper T lymphocyte component into either a Th1 or Th2 effector cell (Itano and Jenkins 2003). Naïve T cells are known to differentiate into immunostimulatory Th1 cells in the presence of the DC secreted pro-inflammatory cytokine IL-12. Alternatively Th0 cells develop into immunosuppressive Th2 cells in the presence of DC secreted immunotolerizing cytokines IL-4 and IL-10. The Th1 lymphocyte subtype secretes inflammatory cytokines such as IL-2 and IFN-γ. These Th1 cells can also support the priming of CD8+ cytotoxic T lymphocytes (CTL), (De Smedt, Butz et al. 2001; Maldonado-Lopez, Maliszewski et al. 2001; Maldonado-Lopez and Moser 2001).

Alternatively, Th2 lymphocytes secrete IL-4, IL-10 and TGF-β (Lutz and Schuler 2002). DCs can also determine the differentiation of naïve T cells into regulatory T cells (Tregs), (Levings, Sangregorio et al. 2001; Roncarolo, Bacchetta et al. 2001; Roncarolo, Levings et al. 2001). Research has shown that Treg cells can suppress DC maturation resulting in maintenance of the immunosuppressive, less mature DC phenotype (Mahnke, Johnson et al. 2007).

The Role of Toll like Receptors in Immune Suppression

To maintain immunological homeostasis, DCs, macrophages and B cells recognize pathogen antigens and autoantigens in the periphery by binding conserved pathogen associated molecular patterns (PAMPS), to trans-membrane Toll-like receptor
(TLR) domains (Takeda and Akira 2001; Takeda and Akira 2003; Wen, Peng et al. 2004). Different PAMPS stimulate specific TLRs activating DC signal transduction pathways through adaptor proteins (e.g., MyD88), leading to synthesis or stimulation of transcriptional activators e.g., nuclear factor kB (NFkB), mitogen activated protein kinases (MAPKs), c-Jun N-terminal kinase (JNK) and interferon regulatory factor 3 (IRF3). These transcriptional activators initiate expression of genes encoding specific cytokines (IL-12, IL-10), costimulatory molecules and chemokines that enable antigen activated DCs to guide the development of naïve Th0 cell into immunoreactive Th1 cells, Th2 immunosuppressive effector cells or regulatory T cell subsets (Kaisho and Akira 2001; Kaisho and Akira 2002). Further, TLR agonists can break immunological tolerance and induce autoimmune diseases such as arthritis (Deng, Nilsson et al. 1999), experimental autoimmune encephalomyelitis (EAE) (Segal, Chang et al. 2000) and diabetes (Lang, Recher et al. 2005). While most TLR agonists are potent adjuvants that promote the development of inflammatory Th1 effector cells, a number of reports show that TLR stimulation may also suppress autoimmune pathogenesis, suggesting a dual role for TLRs in autoimmune diseases (see review by Ehlers and Ravetch, 2007). On occasion, the dual role of TLRs can be demonstrated in the same model, but using different routes of ligand administration. For example, intravenous injection of the TLR9 ligand CpG together with ovalbumin resulted in the induction of tolerance in vivo. However, following subcutaneous injection, the same ligands induced inflammation (Wingender, Garbi et al. 2006). Further, it was demonstrated that TLR2 ligands drive induction of Th2 lymphocyte populations that stimulate immunosuppression, (Hemmi, Takeuchi et al. 2000; Bauer, Kirschning et al. 2001; Kapsenberg 2003; Dillon, Agrawal
et al. 2004; Jarnicki, Conroy et al. 2008; Agrawal S 2003) and modulate the functions of CD4+CD25+ regulatory T cells (Liu, Liao et al. 2006). Recently, examination of TLR2 and TLR4 expression in monocytes from T1D patients showed that TLR2 and TLR4 surface expression and mRNAs were significantly increased in T1D monocytes in comparison with the controls (Devaraj, Dasu et al. 2008). Downstream transcriptional activator and adaptor proteins NF-κB, MyD88, Trif and pIRAK, were also significantly upregulated suggesting DC activation is involved in immune suppression of T1D. Agonists of TLR-4 or TLR-9 can also inhibit autoimmune diseases, such as EAE, arthritis, diabetes, ulcerative colitis and systemic lupus erythematosus (SLE) in mice (Gilkeson, Ruiz et al. 1996; Rachmilewitz, Karmeli et al. 2002; Alyanakian, Grela et al. 2006; Buenafe and Bourdette 2007). Autoreactive T cells involved in EAE and other chronic inflammatory autoimmune diseases are considered to follow defined TLR pathways of DC processing and autoantigen presentation triggered by environment signals (Haverkos, Battula et al. 2003; Ylipaasto, Klingel et al. 2004; Bach 2005). Signaling through TLRs 3 and 7 exacerbates autoimmune diabetes in a mouse model, and the effect is mediated by IFN-α (Lang, Recher et al. 2005).

Dendritic Cell Costimulatory Molecules CD86 (B7-2) and CD83 are Essential for Efficient CD4+ T helper Lymphocyte Activation

Unlike other antigen presenting cells, DCs have a unique capacity to present peptides via MHC class II molecules during maturation, pointing to complex multilevel regulation of antigen presentation by mature DC (Gogolak, Rethi et al. 2003). Activation of naïve Th0 lymphocytes requires engagement of the TCR with the MHC class II - antigen peptide complex presented on the DC plasma membrane surface (Bromley,
DCs can acquire external antigens from the environment; process them within specialized MHC class II rich compartments within the DC as a result of activation and maturation. This initial DC processing induces the transport of antigen peptide loaded MHC class II molecules to the plasma membrane. The capacity of the DC to transport the MHC class II–antigen peptide complex to the cell membrane is dependent upon co-transport of the antigen along with the CD86 co-stimulatory factor protein which together provides efficient activation of CD4+ helper T cells (Inaba, Turley et al. 1998; Inaba, Turley et al. 2000; Turley, Inaba et al. 2000). In addition to antigen specific interactions mediated by the MHC II peptide complex, a second protein interaction involving CD28 originating on T cells and its respective DC ligand CD86 (B7-2), which exists as a monomer on the APC, is required for optimal T cell activation (Carreno and Collins 2002; Collins, Ling et al. 2005).

The cluster of differentiation molecule CD83 is a type-1 membrane glycoprotein of the Ig superfamily expressed by most DCs, including thymic DCs, skin Langerhan’s cells, circulating DCs, interdigitating reticulum cells present in the T cell zones of lymphoid organs and monocyte derived DCs (Zhou, Schwarting et al. 1992; Weissman, Li et al. 1995; Zhou and Tedder 1995; Zhou and Tedder 1995; Zhou and Tedder 1996). The CD83 protein is considered to be a marker of DC maturation. The fact that CD83 biosynthesis is strongly upregulated together with co-stimulatory molecules CD80 and CD86 during DC maturation suggests CD83 plays an important role in the induction of immune responses. Infection studies with herpes simplex virus type 1 (HSV-1) and the inhibition of CD83 mRNA specific transport from the nucleus to the cytoplasm suggests a possible functional role for CD83 (Lechmann, Zinser et al. 2002). The first clear proof
that CD83 is indeed important for DC biology came from recently performed studies employing a soluble form of the extracellular CD83 domain to inhibit DC-mediated T cell proliferation (Lechmann, Kremmer et al. 2002). Additional studies elucidated immunostimulatory as well as regulatory effects of the CD83 molecule (Lechmann, Berchtold et al. 2002; Lechmann, Zinser et al. 2002; Kuwano, Prazma et al. 2007). These studies revealed that CD4+ T cell generation was blocked in CD83−/− knockout mice.

The Role of CD4 and CD8 T Lymphocytes in Type 1 Diabetes Pathogenesis

T cells specifically provide immune protection by activating macrophages and stimulating neutrophil migration to sites of infection, in addition to inducing B cell antibody production (Zhu and Paul 2008).

T lymphocytes exist as a heterogeneous population of CD4+ helper or CD8+ CTLs. However, each T lymphocyte cell type is subdivided into distinct subpopulations. The CD4 lymphocytes are considered to be largely the sole player in the induction of autoimmune annihilation of islet beta cells for the following reasons: 1) in animal models of T1D, CD4 cells were abundantly identified in islet cell infiltrates. 2) Lymphocyte transfer studies suggested that isolation of CD4 T cells from diabetic NOD mice could transfer diabetes into disease free animals (Haskins, Portas et al. 1988). 3) Defective genes involved T1D were mapped within the same region of the MHC for expression of class II polypeptides that specifically interact with CD4. Together, these findings suggested that the autoimmune pathology was caused by rogue T cells escaping into the circulation as a result of faulty positive T cell selection following presentation with diabetogenic peptides (Faustman and Davis 2009). Alternatively, CD8 cells were not
actively considered to play a significant role in T1D because their functions were thought to be limited to fighting foreign antigens such as viruses. However, there is an increasing body of evidence arising from monozygotic twin and NOD mouse studies of T cell education that implicate CD8$^+$ T cells in the pathogenesis of T1D (Faustman, Li et al. 1991; Li, Guo et al. 1994; Faustman and Davis 2009). Other murine and human studies have expanded the role of class I polypeptide presentation of antigens, from providing protection against viral antigens, to protecting against both viral and self antigens. These results directly implicate poorly educated CD8 T cells in the destruction of beta cells expressing self peptides in the class I MHC polypeptide grooves (Faustman, Li et al. 1991; Yan, Shi et al. 1997; Faustman and Davis 2009).

Lymphocyte Subsets Th17 and Tc17 Produce the Inflammatory Cytokine IL-17

Both CD4$^+$ Th17 cells and CD8$^+$ Tc17 cells are two recently described T lymphocyte subsets that are mainly characterized based on their ability to produce the proinflammatory cytokine IL-17. The Th17 lymphocyte subset constitutes a third group of effector T lymphocytes (in addition to Th1 and Th2 cells), found to be associated with autoimmunity (Harrington, Hatton et al. 2005). In addition to the production of IL-17, IL-21, and IL-22 (Bettelli, Oukka et al. 2007), Th17 cells are also distinguished by their expression of the transcription factor retinoic acid, orphan receptor gamma—ROR-$\gamma$t (Ivanov, McKenzie et al. 2006). The Th17 lymphocyte subset is generated when naïve CD4$^+$ T cells are exposed to TGF-$\beta$ and IL-21, or when central memory CD4$^+$ T cells encounter inflammatory cytokines IL-1$\beta$ and IL-6 (Acosta-Rodriguez, Napolitani et al. 2007; Manel, Unutmaz et al. 2008; Volpe, Servant et al. 2008; Yang, Anderson et al. 2008).
The Th17 lymphocyte subset normally functions to control extracellular pathogens, but Th17 cells can also play an important role in human and animal experimental autoimmunity (Bettelli, Oukka et al. 2007). Transfer of islet-specific Th17 cells to NOD mice resulted in diabetes induction, but only after the cells had converted to largely IFN-γ-producing cells (Bending, De La Pena et al. 2009; Martin-Orozco, Chung et al. 2009). The inhibition of Th17 cells, either with neutralizing anti–IL-17 antibodies or with recombinant IL-25, was shown to influence the course of diabetes development in NOD mice (Emamaullee, Davis et al. 2009). Monocytes isolated from the blood of T1D patients were found to spontaneously secrete the proinflammatory cytokines IL-1β and IL-6, which are now known to induce and expand Th17 cell populations (Bradshaw, Raddassi et al. 2009). However, Th17 cells do not in general seem to be independently responsible for the development of autoimmunity. The Th1 cell subset can induce diabetes in the absence of Th17 cells. However, Th17 cells are not sufficient to induce disease in the absence of Th1 cells (Chitnis, Najafian et al. 2001; Bettelli, Sullivan et al. 2004). Recently, it was discovered that Th17 cells also generate large amounts of IL-9, a cytokine normally associated with immuno-suppressive functions produced by Th2 cells (Elyaman, Bradshaw et al. 2009). To further add to the confusion, IL-17 was shown to have a protective effect in T-cell mediated intestinal inflammation (O’Connor, Kamanaka et al. 2009).

The Tc17 lymphocyte subset can also produce IL-17 and usually releases IFN-γ as well (Kondo, Takata et al. 2009). The Tc17 cell phenotype normally functions in the clearance of viral infection (Hamada, Garcia-Hernandez Mde et al. 2009). However Tc17 cells can become diabetogenic if exposed to the inflammatory cytokine IL-23 (Ciric, El-
Little else is known at present about this recently described inflammatory T lymphocyte subset.

Interleukin-10

Interleukin-10, (IL-10) is an anti-inflammatory cytokine with a homodimeric subunit length of 160 amino acids and described immunosuppressive properties (Moore, Vieira et al. 1990; Yoon, Logsdon et al. 2006). IL-10 was identified in APCs including dendritic cell subsets (de Waal Malefyt, Abrams et al. 1991; Sica, Saccani et al. 2000). Mouse knockout studies showed that IL-10 can down-regulate autoimmunity in the intestine and is active in suppression of Crohn’s disease (Grimbaldeston, Nakae et al. 2007; Minderhoud, Samsom et al. 2007). Autocrine production of IL-10 by immature DCs (iDCs), was shown to inhibit synthesis and release of pro-inflammatory cytokines and other molecules (IL-12, TNF-α, IL-6, LTB4, NO, PGE2), and to suppress Th1 lymphocyte activity by down-regulating costimulatory molecule expression on the APC surface (Grutz 2005; Harizi and Gualde 2006). Through inhibition of these endogenous pro-inflammatory mediators, IL-10 is central to maintenance of DCs in an immature state and in the down-regulation of DC mediated inflammatory responses (Harizi and Gualde 2006). In paracrine fashion, IL-10 synthesized by DCs regulates immunity by altering the function of different adjacent cell types. In two studies, IL-10 secreted by iDCs stimulated the development of cognate naïve Th0 cells into Th2 lymphocytes, or suppressor regulatory CD4+ T cells (Romagnani 1998; Romagnani, Kapsenberg et al. 1998; Stassen, Fondel et al. 2004; Stassen, Schmitt et al. 2004). Since IL-10 can partially suppress T1D (Slavin, Maron et al. 2001) in combination with adjuvanted autoantigens
IL-10 may function in an additive or synergistic fashion to enhance multi-subunit vaccines for more effective immune suppression of T1D.

Diabetes - autoantigen Therapy

Immunologic tolerance induced by islet β-cell autoantigens was shown to partially suppress diabetes autoimmunity (Zhang, Davidson et al. 1991) and has been used since then in the treatment of the pathological inflammation associated with organ-specific autoimmune diseases, particularly T1D. This application was developed because the mechanisms of action of the autoantigen may be similar to those operating in the maintenance of immunological homeostasis (Vigouroux, Yvon et al. 2004). Prevention of autoimmune T1D onset or retardation of its progression by repeated oral inoculation with small amounts of pancreatic islet autoantigens has been demonstrated in animal models of autoimmune diabetes (Czerkinsky, Anjuere et al. 1999). Immunotherapy using major β-cell antigens such as insulin, GAD, or heat shock protein (hsp60), was shown in a variety of studies to delay or prevent T1D onset (Elliott, Qin et al. 1994; Tisch and McDevitt 1996; Atkinson and Leiter 1999). Oral inoculation with small amounts of islet autoantigens was shown to induce self-tolerance through IL-4 and Stat 6 activation of CD4+ Th2 and Th3 regulatory T cells that down-modulate autoreactive effector T cell inflammation at close proximity e.g., “bystander suppression”, (Hommann, Schotte et al. 2001; Weiner 2001). Protection against T1D was shown to be associated with Th3 regulatory lymphocyte secretion of TGF-β in prediabetic NOD mice gavaged with insulin, (Zhang, Davidson et al. 1991). Further, oral autoantigen inoculation generated partial diabetes suppression in patients (Chaillous, Lefevre et al. 2000; Millington,
Mowat et al. 2004). In several related Th1 cell mediated autoimmune diseases, collagen-induced arthritis (Thompson and Staines 1986) and encephalomyelitis (Higgins and Weiner 1988), disease suppression was observed following prolonged feeding of small amounts of specific autoantigens. The induction of multiple sclerosis in animals was not only halted but reversed by feeding small amounts of myelin basic protein (MBP) suggesting that this type of oral autoantigen therapy can suppress and even aid in recovery from autoimmunity (Bitar and Whitacre 1988).

Incidentally, most individual autoimmune diabetes vaccination strategies were developed in two mammalian (rodent) models that spontaneously develop T1D, the NOD mouse and the BioBreeding (BB) rat. These animal models have allowed detailed exploration of the altered communication between DCs of the innate arm of the immune system that underlies development of pancreatic β-cell-reactive effector T cells of the adaptive portion of the immune system. However, the animal model that has emerged as the most frequently employed for pre-clinical evaluation of prophylactic and therapeutic strategies for treatment of T1D has been the NOD mouse, which has an etiology of autoimmune diabetes similar to that of humans such as the influence of sex on disease incidence (Anderson and Bluestone 2005).

Preference for the NOD mouse model is based on a better defined genome, more monoclonal reagents for the analysis of immune system components and lower animal maintenance costs. So much preference has been placed on the NOD mouse for diabetes studies that other animal models frequently remain untested and potential differences in the NOD model as compared with humans are often neglected prior to extrapolations made to patients. More than 125 individual therapeutic strategies have been reported to
have a therapeutic effect on diabetes prevention in the NOD mouse (Atkinson and Leiter 1999). However, most of these therapies have shown only partial suppression of the disease.

In contrast to diabetes prevention, amelioration of diabetes hyperglycemia following disease onset is even more difficult to achieve. Less than a dozen immunomodulatory protocols have been reported to have a detectable therapeutic effect (Anderson and Bluestone 2005). Importantly, several of these therapies subsequently showed partial efficacy in clinical trials (Raz, Elias et al. 2001; Herold, Hagopian et al. 2002; Keymeulen, Vandemeulebroucke et al. 2005). Based on the partial success in prevention of T1D onset in NOD mice, this mammalian model has often been considered to be an indicator of the potential for individual therapies in humans.

Cholera Toxin B Subunit (CTB) Enhances Autoantigen-Mediated Immune Tolerance

Cholera enterotoxin (CT) is an oligomeric protein produced in nature by the Gram-negative bacterium *Vibrio cholerae*. The toxin causes excretion of a high volume of dilute (“rice water”) diarrhea initiated in the upper part of the small intestine. SDS-PAGE analysis showed that CT consists of a single large A subunit (CTA) of approximately 27 kDa and a pentameric B subunit (CTB) with an approximate monomer molecular weight (MW) of 10.6 kDa (Lonnroth and Holmgren 1973). The CTA subunit can be subdivided into CTA1 and CTA2 subunits linked by a disulfide bond. The CTA1 subunit was found to be responsible for CT toxicity (Sattler and Wiegandt 1975). The assembly of CT structures revealed that the toxic CTA1 subunit contained ADP-ribosylating activity, while the helical CTA2 fragment was found to be responsible for embedding the CTA1 subunit.
into the center of the doughnut shaped CTB pentameric oligomer (Sixma, Kalk et al. 1993). In addition, the CTB subunit, held together by hydrogen bonds and salt bridges was shown to bind to ganglioside GM1[Gal(β1-3)galNac(β1-4)(NeuA-c(α2-3)Gal(β14)Glc]→ceramide (Merritt, Sarfaty et al. 1995) an anchor molecule embedded in the mammalian epidermal cell membrane (Holmgren, Lonnroth et al. 1973; Holmgren, Lonnroth et al. 1975) (Fig. 1.4). Cholera toxin was shown to bind and infect a variety of somatic cells in vivo, especially intestinal epithelial cells, through high affinity binding of the toxin to its cell surface receptor GM1 ganglioside (Holmgren, Lindholm et al. 1974; Holmgren, Lonnroth et al. 1975; van Heyningen 1976; van Heyningen and King 1976). However, only epidermal cells in the Go/G1 phase of the cell cycle were shown to both bind and internalize CT.
Figure 1.4. Crystal structure of cholera toxin.
The heterodimeric CTA protein subunit (blue) is composed of two polypeptide chains, CTA1 (22 kDa) and CTA2 (5 kDa), linked by a single disulfide bond. The enzymatically active CTA1 peptide is the (toxic) mono-ADP-ribosyltransferase subunit while the CTA2 helical peptide links the CTA1 subunit to the pentameric CTB subunits. The cholera toxin B subunit (10.6 kDa) is composed of five identical polypeptide subunit chains (yellow, purple, red, orange and turquoise), each with membrane receptor GM1ganglioside binding capacity.
CT binds preferentially and almost exclusively to GM1 ganglioside (Sixma, Kalk et al. 1993; Sanchez and Holmgren 2008). Cholera toxin secretion in bacteria involves transport across the outer membrane through a CT secretion system known as the extracellular protein secretion system (Eps) (Camberg, Johnson et al. 2007). The energy for secretion is provided by EpsE, a cytoplasmic ATPase, that forms a complex with other secretory proteins to transfer CT across the periplasmic compartment (Camberg and Sandkvist 2005). This transfer is believed to be facilitated by the outer membrane component of the Eps, EpsD, which induces opening of the channel and subsequent secretion (Davis, Lawson et al. 2000). This protein transfer system moves CT from the periplasm where its subunits are assembled, across the membrane and into the extracellular environment (Hirst, Sanchez et al. 1984; Hardy, Holmgren et al. 1988). In order to mediate its toxic activity, CT binds with high affinity to GM1 ganglioside in lipid rafts on the epidermal cell surface of the lumen of the small intestine. The high binding affinity of CTB to ganglioside GM1 is due to the contribution of a single amino acid (Gly33) on the neighboring CTB monomer to the GM1 binding site on an adjacent CTB monomer (Merritt, Sarfaty et al. 1994). Subsequently, the crystal structure of CT revealed that Tyr12 on the CTB monomer along with Gly33 and Trp88 on the adjacent monomer are critical for CT-GM1 interaction (Jobling and Holmes 2002).

The binding of CT via its CTB subunits to GM1 permits toxin endocytosis through caveolin-coated and clathrin-coated vesicles (Chinnapen, Chinnapen et al. 2007). In addition, CT was shown to enter cells through both Arf6 dependent and non-Arf6 dependent pathways, which still remain unidentified (Chinnapen, Chinnapen et al. 2007).
Interestingly, blocking all known endocytic pathways does not appear to alter the toxicity of CT within the cell (Massol, Larsen et al. 2004). The toxin is transported not only to endosomes but also to the endoplasmic reticulum (ER) via Golgi retrograde transport mechanisms (Sandvig and van Deurs 2002). An endoplasmic reticulum retention motif (KDEL) is located near the C terminus of the CTA chain. This motif allows the toxin to interact with the KDEL receptor which permits recycling of ER components from the trans-Golgi network (TGN), back to the ER (Spooner, Smith et al. 2006). Endocytosis of the toxin results in CTA1 subunit induction of adenylate cyclase. The up-regulation of adenylate cyclase activity occurs through CTA stimulation of ADP ribosylation of the adenylate cyclase Gsα subunit (O'Neal, Jobling et al. 2005). Increased intracellular cAMP concentrations result in an imbalance in electrolyte influx into the cell due to decreased sodium uptake by enterocytes and an increase in anion efflux from the cells. The decrease in sodium intake in addition to the extrusion of anions and bicarbonates causes water to be excreted from the cell into the lumen of the intestine. Ultimately, large amounts of water and electrolytes are lost from the intestinal epithelium resulting in severe bouts of “rice water” diarrhea. In addition, there is fluid loss from the intestine of up to 2 liters per hour leading to dehydration and death, usually by stroke in patients in which rehydration therapy is unavailable.

The Cholera Toxin B subunit (CTB) Functions as a Carrier and Vaccine Adjuvant

Enhanced immunogenic and adjuvant properties of microbial holotoxin B subunits such as CTB and heat-labile enterotoxin (LTB) from *E. coli* have been widely reported upon in a number of recent studies (Sun, Holmgren et al. 1994; Czerkinsky, Sun
et al. 1996; Guidry, Cardenas et al. 1997; Porgador, Staats et al. 1998; Sun, Xiao et al. 2000; Eriksson and Holmgren 2002). The intact CT holotoxin was also found to be a potent mucosal immunogen. The presence of potent CT toxic effects, CT resistance to proteases and bile salts, as well as the high affinity of CT binding to ganglioside GM1 and corresponding enzymatic ADP-ribosylating activity may contribute individually or together to establish the powerful immunostimulatory activity of CT (Sanchez and Holmgren 2008). Despite its strong immunogenic activity, and its wide use in animal vaccination protocols, the ability of CT to induce persistent inflammation has delayed application of the holotoxin as an adjuvant for stimulating immune responses in human vaccines.

Despite this present drawback, oral CTB cholera vaccines have proved to be effective against cholera toxin (Arakawa, Yu et al. 1998; Lucas, Deen et al. 2005). Surprisingly, in addition to its known capacity to induce a pro-inflammatory response, oral administration of CTB subunit coupled with an autoantigen was shown to induce a state of immunological tolerance (George-Chandy, Eriksson et al. 2001; D’Ambrosio, Colucci et al. 2008). In early studies, oral delivery of CTB conjugated to specific autoantigens was shown to enhance autoantigen mediated protection of mice against several autoimmune diseases including autoimmune encephalomyelitis (Sun, Xiao et al. 2000), autoimmune chondritis (Kim, Cheng et al. 2001), and uveitis (Phipps, Stanford et al. 2003). Further, CTB-autoantigen conjugates were shown to substantially suppress T1D in NOD mice (Bergerot, Ploix et al. 1997; Arakawa, Yu et al. 1998). Results of diabetes studies indicated that CTB-autoantigen conjugates reduced IFN-γ production and the migration of Tr1 regulatory T cells into pancreatic islets (Roncarolo, Levings et
al. 2001; Aspord and Thivolet 2002). Linkage of CTB to an autoantigen was shown to provide up to a 10,000-fold reduction in the amount of autoantigen required for generating immuno-tolerance (Arakawa, Chong et al. 1998; George-Chandy, Eriksson et al. 2001).

Mechanisms underlying CTB-autoantigen modulated tolerance were shown to include inhibition of DC maturation, autoreactive T cell development and/or stimulation of Th2 and Foxp3 regulatory T cell proliferation and activation (Marinaro, Staats et al. 1995; Lavelle, McNeela et al. 2003; Lavelle, Jarnicki et al. 2004). In other studies, incubation of immature DCs with CTB was shown to induce DC maturation in experimental tumor models (George-Chandy, Eriksson et al. 2001; Isomura, Yasuda et al. 2005). Morphological changes in DCs incubated with CTB included cell enlargement, elongation of DC dendrites and increased migration of DCs into draining lymph nodes as well as increased expression of the B7-2/CD86 co-stimulatory molecule (Isomura, Yasuda et al. 2005). Further, the mucosal administration of CTB conjugated to autoantigens was shown to mediate synthesis of T cell cytokines in response to the antigen or autoantigen complex. In experimental allergic encephalitis studies, the secretion of proinflammatory cytokines IL-12, IFN-γ, and TNF-α were significantly reduced while T cell expression of TGF-β was increased in animals treated with CTB conjugated to MBP (Sun, Xiao et al. 2000). Similarly, immunosuppressive cytokine secretion including increased IL-10 secretion was observed after oral administration of CTB conjugated to insulin, resulting in suppression of diabetes onset in NOD mice (Bergerot, Ploix et al. 1997; Ploix, Bergerot et al. 1999; Aspord and Thivolet 2002) (Fig.1.5). Based on the recent findings that inflammatory Th17 lymphocytes were
implicated in autoimmune disease pathogenesis, conjugation of CTB to myelin oligodendrocyte glycoprotein (MOG) delivered together with Complete Freund’s Adjuvant (CFA) resulted in suppression of lymphocyte IL-17 secretion (Sun and Holmgren unpublished results).

CTB May Stimulate Tolerance through Binding DC Toll-like Receptors

To maintain immunological homeostasis, DCs recognize pathogen antigens and autoantigens in the periphery. Immunological recognition is achieved by binding conserved PAMPS, to trans-membrane TLR domains (Takeda and Akira 2001; Takeda, Kaisho et al. 2003; Wen, Peng et al. 2004). Different PAMPS stimulate specific TLRs activating DC signal transduction pathways through adaptor proteins (e.g., MyD88), which lead to synthesis or stimulation of transcriptional activators e.g., NFκB, MAPKs, JNK and IRF3 as mentioned earlier. These transcriptional activators initiate expression of genes encoding specific cytokines (IL-12, IL-10), costimulatory molecules and chemokines that enable antigen activated DCs to guide the development of naïve Th0 cell into immunoreactive Th1 cells, Th2 immunosuppressive effector cells or regulatory T cell subsets (Kaisho and Akira 2001; Kaisho and Akira 2002). While most TLR agonists are potent adjuvants that promote the development of inflammatory Th1 effector cells, a number of reports show TLR2 ligands drive induction of Th2 lymphocyte populations that stimulate immunosuppression (Hemmi, Takeuchi et al. 2000; Kapsenberg 2003; Wen, Peng et al. 2004; Jarnicki, Conroy et al. 2008; Agrawal S 2003). Downstream transcriptional activator and adaptor proteins NFκB, AP-1 MyD88, Trif and pIRAK, were also significantly upregulated suggesting DC activation is involved in immune
suppression of T1D. In parallel studies, the *E. coli* enterotoxin B subunit (LTB, which is approximately 80% homologous in amino acid sequence to CTB), was shown to stimulate TLR2, resulting in regulatory T cell proliferation, IL-10 secretion and induction of Th2 anti-inflammatory lymphocyte development (Hajishengallis, Tapping et al. 2005; Sutmuller, den Brok et al. 2006; Sutmuller, Morgan et al. 2006). The structural homologies between CTB and LTB proteins and their amino acid sequences suggest similarities in mechanisms underlying inhibition of DC maturation (Marinaro, Staats et al. 1995; Lavelle, McNeela et al. 2003; Lavelle, Jarnicki et al. 2004).

**Conclusion**

The cellular and molecular mechanisms responsible for adjuvant enhanced autoantigen mediated suppression of autoimmune responses remain largely obscure. The fusion of CTB subunits with autoantigens that in general stimulate immune tolerance against the linked autoantigen was found to exert a particularly useful effect in the prevention of organ-specific autoimmune disease onset (Arakawa, Yu et al. 1998; Arakawa, Yu et al. 1999; Kobayashi, Abiru et al. 2007). However, in several studies, fusion of the toxin B subunit to specific autoantigens such as carcinoembryonic antigen (CEA) or prostate specific antigen (PSA) resulted in stimulation of an inflammatory rather than an immunosuppressive response (Facciabene, Aurisicchio et al. 2007; Fensterle, Bergmann et al. 2008).

To understand how multi-component vaccines can function to prevent diabetes onset, it will be important to improve our understanding of mechanisms underlying T1D onset. One of the first steps required to reach this goal, will involve identification of
vaccine interactions with cells of the innate immune system involved in initial vaccine processing i.e., DCs. It will be necessary to determine how DC processing of adjuvant-autoantigen fusion proteins results in the initiation of immuno-reactive or immuno-suppressive T cell populations capable of inhibiting the development of autoimmune T cell responses.

The experimental studies presented in this dissertation emphasize the focus of our laboratory in elucidation of early mechanisms underlying specific DC immune responses to inoculation with CTB subunit-diabetes autoantigen fusion proteins. The experimental results help to identify the role of protein adjuvants such as CTB linked to diabetes autoantigens on DC maturation and how these immunomodulated DCs influence the morphogenesis and proliferation of immunostimulatory Th1 and immunosuppressive Th2 and/or regulatory T cell subsets to enhance the immunological suppression of diabetes onset \textit{ex vivo} in humans. Mechanisms underlying multi-component vaccine stimulation of immunological tolerance must be more completely elucidated in order to fully harness this promising combinatorial vaccination strategy for transfer into safe and effective clinical therapy in T1D patients.
Figure 1.5. Adjuvant-autoantigen suppression of type 1 diabetes pathogenesis
In this cartoon model, immature dendritic cells (iDC) in the gut periphery take up CTB-INS/GAD fusion proteins from the intestinal lumen. Alternatively, microfold (M)-cells overlaying the Peyer’s patches take up the fusion proteins by endocytosis, where they are then delivered to antigen presenting cells, particularly dendritic cells and macrophages that reside in the lamina propria. Autoantigens, made more immunoreactive by linkage to the CTB adjuvant, modulate iDC processing and maturation favoring DC secretion of IL-10 and suppression of IL-12 secretion. The anti-inflammatory cytokine IL-10 directs cognate Th0 cell development into immunosuppressive Th2 or iTreg (Th3 or Tr1) cell phenotypes. CTB-INS/GAD may also activate CD4+CD25+ native regulatory T cell (nTreg) synthesis of transcriptional activator protein FOXP3, leading to further immune suppression activity.
Figure adapted from: Afzali B. et al. (2007), Iwasaki A. and Kelsall, B.L. (2000), and a 2008 NIH R01 application submitted by W. Langridge and I. Fodor to the National Institute of Digestive and Diseases of the Kidney (NIDDK) entitled, Immunostimulation of Autoantigen Mediated Tolerance.


Weissman, D., Y. Li, et al. (1995). "Three populations of cells with dendritic morphology exist in peripheral blood, only one of which is infectable with human immunodeficiency virus type 1." Proc Natl Acad Sci U S A 92(3): 826-830.


CHAPTER TWO

SUPPRESSION OF DENDRITIC CELL ACTIVATION BY DIABETES AUTOANTIGENS LINKED TO THE CHOLERA TOXIN B SUBUNIT

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List of Abbreviations: DC: dendritic cell; TLR: Toll-like receptor; T1D: type 1 diabetes
PMA: Phorbol myristate acetate; LPS: lipopolysaccharide
Abstract

Antigen presenting cells (APC); specifically dendritic cells (DCs) are a focal point in the delicate balance between T cell tolerance and immune responses contributing to the onset of type I diabetes (T1D). Weak adjuvant proteins like the cholera toxin B subunit when linked to autoantigens may sufficiently alter the balance of this initial immune response to suppress the development of autoimmunity. To assess adjuvant enhancement of autoantigen mediated immune suppression of T1D, we examined the cholera toxin B subunit (CTB)-proinsulin fusion protein (CTB-INS) activation of immature dendritic cells (iDC) at the earliest detectable stage of the human immune response. In this study, incubation of human umbilical cord blood monocyte-derived immature DCs with CTB-INS autoantigen fusion protein increased the surface membrane expression of DC toll-like receptor (TLR-2) while no significant upregulation in TLR-4 expression was detected. Inoculation of iDCs with CTB stimulated the biosynthesis of both CD86 and CD83 co-stimulatory factors demonstrating an immunostimulatory role for CTB in both DC activation and maturation. In contrast, incubation of iDCs with proinsulin partially suppressed CD86 co-stimulatory factor mediated DC activation, while incubation of iDCs with CTB-INS fusion protein completely suppressed iDC biosynthesis of both CD86 and CD83 costimulatory factors. The incubation of iDCs with increasing amounts of insulin (INS) did not increase the level of immune suppression but rather activated DC maturation by stimulating increased biosynthesis of both CD86 and CD83 costimulatory factors. Inoculation of iDCs with CTB-INS fusion protein dramatically increased secretion of the immunosuppressive cytokine IL-10 and suppressed synthesis of the pro-inflammatory cytokine IL12/23 p40 subunit protein.
suggesting that linkage of CTB to INS may play an important role in mediating DC guidance of cognate naïve Th0 cell development into immunosuppressive T lymphocytes. Taken together, the experimental data suggests that TLR-2 plays a dominant role in CTB mediated INS inhibition of DC induced T1D onset in humans. Further, fusion of CTB to the autoantigen was found to be essential for enhancement of immune suppression as co-delivery of CTB and INS did not significantly inhibit DC costimulatory factor biosynthesis. The experimental data presented supports the hypotheses that adjuvant enhancement of autoantigen mediated suppression of islet beta cell inflammation is dependent on CTB stimulation of DC TLR-2 receptor activation and co-processing of both CTB and the autoantigen in the same DC.

**Introduction**

Insulin dependent diabetes mellitus, or Type 1 diabetes (T1D), is the most destructive metabolic disease of children. T1D is caused by autoreactive lymphocyte destruction of insulin-producing islet β-cells of the pancreas (Eisenbarth 1986; Tisch and McDevitt 1996). The progressive loss of islet β-cell function results in an increasing deficiency of insulin production resulting in elevated blood sugar levels (hyperglycemia). The inability to transfer glucose from the blood into the cells of the body results in increased levels of cellular oxidative stress which leads to chronic inflammation throughout the body resulting in an increased and premature risk for secondary neural and circulatory health problems, including amputation of extremities, blindness, heart attack and stroke (Libby, Nathan et al. 2005). Young T1D patients must inject insulin several times a day for the rest of their lives or risk diabetic shock and death (Lodinova-
The first step in diabetes-mediated breakdown of immunological homeostasis leading to islet β-cell mortality is initiated by autoantigen-stimulated maturation of antigen-presenting cells (APCs), largely dendritic cells (DC). Maturation of DCs induces the development of autoreactive CD8+ cytotoxic T cells (CTL) and CD4+ T helper (Th1) cells, as well as B cell production of autoantigen specific antibodies (Atkinson and Maclaren 1994; Tisch and McDevitt 1996; Han, Serra et al. 2005; Tang, Adams et al. 2006). Following autoreactive CD4+ Th1 cell infiltration of pancreatic islets in non-obese diabetic (NOD) mice, autoreactive Th1 lymphocytes were shown to secrete the inflammatory cytokines IFN-gamma and IL-2. These diabetes autoantibodies are known to stimulate macrophage and CTL secretion of oxidative compounds (NO, O₂, H₂O₂) in addition to inflammatory cytokines (IL-1 beta, TNF-alpha, TNF-beta, IFN-gamma) (Atkinson and Maclaren 1994; Han, Li et al. 2005; Tang, Adams et al. 2006). The persistence of these immune responses induces chronic pancreatic inflammation (insulitis), which results in the induction of apoptosis of approximately 90% of the islet insulin-producing β-cells leading to insufficient levels of insulin production (Piccinni, Beloni et al. 1998). A variety of immune cell types including B cells, dendritic cells (DCs), macrophages and natural killer (NK) cells were shown to be involved in the onset of diabetes pathogenesis (Kent, Chen et al. 2005; Wang, Zhang et al. 2005; Yoon and Jun 2005; Cardell 2006; Silveira and Grey 2006; Tian, Zekzer et al. 2006; Tian, Hao et al. 2009). Specifically, DCs were shown to play a primary role in antigen priming of naïve Th cells (Th0) and in the modulation of their development into autoreactive Th1 lymphocytes or immunosuppressive Th2 cells critical for maintenance of immunological
homeostasis (Pulendran, Smith et al. 1999; Pulendran, Kumar et al. 2001; Itano, McSorley et al. 2003). Immuno-cytochemical analyses showed that oral inoculation results in autoantigen uptake through M cells of the intestinal epithelium into peripheral DCs via several routes that may aid in the establishment of immune suppression (Yoon and Jun 2005). Autoantigens (autoantigen (autoAg)) are taken up and processed by immature DC subsets (Figdor, de Vries et al. 2004). Following DC activation by autoantigens, the DCs migrate to adjacent lymph nodes, where they present antigen peptides on major histocompatibility complex class II (MHCII) receptors, synthesize co-stimulatory molecules and secrete IL-12 which guides the development of naïve cognate Th0 cells into Ag-specific inflammatory Th1 lymphocytes. In contrast, oral inoculation with small amounts of autoantigen was shown to induce DC production of the anti-inflammatory cytokine IL-10 which stimulates the development of naïve Th0 lymphocytes into anti-inflammatory Th2 lymphocytes, or alternatively into IL-10 or TGF-beta producing CD4+CD25+ Tr1 or Th3 regulatory T cells (Kapsenberg 2003; D'Ambrosio, Colucci et al. 2008). Thus, interactions between autoantigens, DCs and T cells in the gut-associated lymphoid tissues, may dictate the onset of inflammatory or tolerogenic outcomes following initial autoantigen (autoAg) presentation. In addition, DCs residing in lymphoid follicles and the Peyer’s patches, shown to synthesize IL-10, were found to down-regulate Th1 cell mediated autoimmunity (Steinbrink, Wolfl et al. 1997). Further, immature or peripheral DCs (iDCs), that displayed low levels of co-stimulatory molecule expression and that secreted cytokine IL-10, were shown to remain in the periphery and were found to induce Th2 lymphocyte mediated immunological tolerance (Rissoan, Soumelis et al. 1999; Liu, MacKenzie-Graham et al. 2001; Holmgren,
Adamsson et al. 2005; Li, Zhong et al. 2006). In an alternative set of experiments, the addition of IL-12 to iDCs induced autoreactive Th1 cell morphogenesis and accelerated T1D in NOD mice (Trembleau, Germann et al. 1995). In composite, the available experimental data suggests that initial stages of T1D progression may be largely under DC control and may set the stage for anti-inflammatory or inflammatory disease outcomes (Shinomiya, Fazle Akbar et al. 1999).

Oral administration of autoantigens has shown promise for prevention of spontaneous autoimmune diabetes (Zhang, Davidson et al. 1991; Trentham, Dynesius-Trentham et al. 1993). However, the need for repeated autoantigen administration over an extended period of time poses a limitation to such therapy. Further, a low efficiency of immune suppression was reported in previously sensitized hosts (Arakawa, Yu et al. 1998). These limitations were largely overcome through application of the nontoxic B subunit of the cholera enterotoxin (CTB) from Vibrio cholerae. The CTB molecule was shown to be a strong immunomodulator for induction of oral tolerance when used as a carrier molecule for conjugated autoantigens (Sun, Holmgren et al. 1994; Sun, Rask et al. 1996; Sun, Li et al. 2000; Sun, Xiao et al. 2000). The bacterial AB enterotoxin from Vibrio cholerae, cholera toxin (CTX) contains a toxic ADP-ribosyltransferase subunit A1 (CTA1), linked through a small helical (A2) peptide to a pentamer of non-toxic B carrier subunits (CTB). The CTB subunits were shown to be required for binding the toxin to monosialoganglioside receptor molecules embedded in gut epithelial cell membranes facilitating entry of the holotoxin into the cell (Eriksson and Holmgren 2002). The CTB subunit was shown to bind specifically to GM1-ganglioside, a receptor molecule found in common on the membrane of most types of epidermal cells. Thus, CTB can provide an
efficient trans-mucosal carrier molecule for autoantigen induction of peripheral tolerance (Sun, Holmgren et al. 1994; Shreedhar, Kelsall et al. 2003).

In previous studies, oral delivery of CTB conjugated to specific autoantigens was shown to enhance autoantigen mediated protection of mice against several organ-specific autoimmune diseases including autoimmune encephalomyelitis (Sun, Xiao et al. 2000) autoimmune chondritis (Kim, Cheng et al. 2001) and uveitis (Phipps, Stanford et al. 2003). In addition, CTB-INS conjugates were shown to substantially suppress diabetes in NOD mice (Bergerot, Ploix et al. 1997; Arakawa, Yu et al. 1998). The observed suppression of diabetes onset was associated with a reduction in Th1 cell IFN-γ production and the migration of Tr1 regulatory T cells into pancreatic islets (Roncarolo, Levings et al. 2001; Aspord and Thivolet 2002). Further, the fusion of CTB to insulin was shown to provide up to a 10,000-fold reduction in autoantigen amounts required for immuno-tolerization (Arakawa, Chong et al. 1998; George-Chandy, Eriksson et al. 2001). Mechanisms underlying CTB modulated immune suppression of T1D may include the inhibition of DC maturation, inhibition of autoreactive T cell development and/or induction of Th2 and inducible T regulatory cell (iTreg) proliferation and activation (Marinaro, Staats et al. 1995; Lavelle, McNeela et al. 2003; Lavelle, Jarnicki et al. 2004).

Recent immunotherapy and vaccination strategies strongly target receptors that mediate immune cell activation. Pathogen recognition receptors, especially TLRs expressed by APCs, have received increasing attention because activation of innate immunity through pathogen protein, nucleic acid and lipopolysaccharide (LPS) pattern recognition has been increasingly identified as an essential first line of immunological defense (Hemmi, Takeuchi et al. 2000; Schnare, Barton et al. 2001; Takeda, Kaisho et al.
In general, TLRs interact with a variety of microbial structures widely expressed by fungi, bacteria, protozoa and viruses conferring a relatively high degree of specificity to the immune response (Takeda, Kaisho et al. 2003). Recent developments support the concept that activation of immunity by microbial molecules may involve cooperative interaction with multiple host receptors within the membrane lipid raft (Triantafilou, Miyake et al. 2002; Beutler, Jiang et al. 2006; Hoebe, Jiang et al. 2006). That is, TLRs are usually present as preformed homodimers with the exception of TLR2, which preferentially forms heterodimers with either TLR1 or TLR6 (Akira and Takeda 2004). Toll like receptors TLR2/TLR1 and TLR2/TLR6 were shown to be activated in response to agonists such as lipoteichoic acid and lipoproteins, while other APC surface and internal TLRs respond to a variety of bacterial and virus DNA and LPS immunostimulatory molecules (Akira and Hemmi 2003; Takeda, Kaisho et al. 2003; Roger, Miconnet et al. 2005; Kanzler, Barrat et al. 2007). Recently, the type II heat-labile enterotoxin from E. coli which has an AB₅ subunit structure similar to cholera toxin was found to stimulate cytokine release in mouse and human cells through interactions with TLR2. However, up to the present, the mechanism of CTB mediated TLR activation is only poorly understood. An increased understanding of the initial interactions between CTB and its fusion proteins with TLRs is predicted to improve our understanding of mechanisms by which these molecules exert their immunomodulatory activities.

The objectives of this study are to clarify mechanisms underlying early events in CTB-INS vaccine induction of immunological tolerance leading to the suppression of T1D through analysis of CTB-proinsulin autoantigen induced inhibition of human immature DC activation and maturation leading to induction of Th0 lymphocyte
development into inflammatory Th cells resulting in chronic pancreatic islet inflammation and the death of insulin producing islet β-cells. Examination of CTB and CTB-INS fusion protein interactions with DC membrane TLRs will provide information on whether they or other membrane receptors may play a role in the initiation of adjuvant-autoantigen mediated DC activation and maturation. Through analysis of CTB-autoantigen fusion protein interactions with immature DCs we may be able to gain a clearer understanding of how CTB-INS fusion proteins interact with DCs to suppress the onset of T1D.

**Materials and Methods**

**Construction of Bacterial Plasmids Containing CTB-INS**

A gene encoding approximately 258bp of human proinsulin (INS) was physically linked to the carboxyl-terminus (309bp) of CTB to generate the fusion gene CTB-INS. The cholera toxin B subunit–autoantigen fusion gene CTB-INS was cloned into the (A) configuration of the *E. coli* expression vector pRSET (Invitrogen™, Carlsbad, CA), under control of the bacteriophage T7 promoter in order to achieve high levels of transgene expression. The pRSET vector also contained an oligonucleotide encoding 6 histidines immediately upstream of the CTB permitting isolation of the transgene product. The CTB-INS fusion gene (567bp) was cloned between the BamH1/BglI and EcoR1 sites flanked by a termination sequence and a poly (A) adenylation sequence in the 3’ region of the transgene. Selective clones were assessed by DNA sequence analysis to confirm the in-frame linkage of CTB and Insulin DNA fragments (Fig. 2.1A). The expression vector pRSET-CTB-INS containing the gene encoding the CTB-INS fusion
protein was introduced into *E. coli* producer strain BL21 (DE3)pLysS (Invitrogen, Carlsbad, CA) for nickel affinity column isolation of the recombinant protein (Carter, Yu et al. 2006).

**Synthesis and Isolation of CTB-INS Fusion Proteins**

The CTB-INS transformed *E. coli* strain BL-21, was grown in 250 ml Luria Broth (LB) medium containing ampicillin (100mg/ml). While still in log phase of growth, CTB-INS protein synthesis was stimulated by addition of 90 mg isopropyl β-D-1thiogalactopyranoside (IPTG), (Sigma Chemical Co. St. Louis, MO), to the bacterial culture. After 6 hr continued growth at 37°C, the bacterial culture was transferred into 50 ml polystyrene Oakridge tubes and the cells harvested by centrifugation in a SA-600 rotor for 10 min, at 5,000 rpm and 4°C, in a Sorvall RC5B centrifuge. The cell pellet was resuspended in 1.0 ml/tube of 10 mM HEPES buffer (pH 7.5), containing 100 mM imidazole. The cells were disrupted by sonication at 3 x 10 sec bursts at 10 W, with a Sonic 60 Dismembrator, (Fisher Scientific, and Sunnyvale, CA). The CTB-INS protein was isolated and purified from the bacterial homogenate using a Maxwell Model 16 robotic protein purification system (Promega Inc.™), according to the protein isolation protocol provided by the manufacturer (Promega Inc., Madison, WI). In order to obtain a pure protein product, the robot employs electromagnetically charged Magne-His Nickel-Iron alloy particles with an affinity for the 6-HIS tag linked to the N terminus of the recombinant CTB-INS fusion protein. Imidazole was removed from the protein mixture by dialysis of the preparation against 2 x 1.0 Liter, 10 mM HEPES buffer (pH 7.5), for 4 hr at 4°C. The purity of the isolated CTB-INS protein (23.4kDa) was determined by electrophoretic mobility analysis.
in a 12% polyacrylamide gel in comparison with protein molecular weight standards (Fig. 2.1B). The purified CTB-INS protein was stored at -20°C until further use.

Isolation and Culture of Monocyte-Derived DC from Human Cord Blood

Monocyte-derived dendritic cells (MDDC) were prepared from freshly collected human umbilical cord blood (Appendix 1). The leukocyte fraction of approximately 50 ml of cord blood obtained from normal healthy placenta donors (by LLU IRB approved protocols), was separated from red blood cell and platelet fractions by Ficoll-paque density gradient centrifugation for 30 min at 2,000 rpm @ 4°C, in a Beckman Coulter Allegra X-15R centrifuge equipped with an SX4750 rotor (Appendix 2). The CD14+ monocytes were obtained from the total lymphocyte fraction by incubation with anti-CD14 PE (Phycoerythrin) for 10 min @ 4°C followed by incubation with anti-PE magnetic microbeads for 15 min at 4°C. The cells were separated magnetically by passing them through the MACS column as described by the manufacturer (Miltenyi Biotech, Auburn, CA) (Devaraj, Glaser et al. 2006; Devaraj, Dasu et al. 2008). The purity of the monocyte fraction was determined by flow cytometry in a BD FACSCalibur flow cytometer (BD Biosciences, San Jose, CA).

The isolated monocytes were cultured in 6 cm non-pyrogenic polystyrene culture plates in RPMI 1640 culture medium (Mediatech Inc. Manassas, VA, USA) in a humidified atmosphere of 5% CO₂ at 37 °C (Preprotech, Rocky Hill, NJ) (Appendix 4). The medium was supplemented with 10% fetal bovine serum (FBS), 1 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 ng/ml human recombinant GMCSF, and 10 ng/ml human recombinant IL-4 (ProSpec-Tany TechnoGene, Rehovot Science Park, Israel). The monocyte cell culture was fed every 2 days by gentle replacement of 50% of the medium.
Figure 2.1 Construction of the CTB-INS Fusion Gene and Isolation of the Gene Product. Panel A shows the plasmid map of the E. coli expression vector pRSET A (Invitrogen™, Carlsbad, CA), carrying the CTB-INS fusion gene. The cholera toxin B subunit–proinsulin autoantigen gene fusion, CTB-INS (567bp), was cloned into pRSET (A) between the BamH1/Bgl I and EcoR1 sites. The expression vector pRSET-CTB-INS with the CTB-INS coding sequence under control of the bacteriophage T7 promoter also contains an oligonucleotide region encoding 6 histidine amino acid residues immediately 5’ upstream of the CTB DNA sequence. The recombinant plasmid was introduced into the E. coli recipient strain BL21 (DE3) pLysS, for CTB-INS fusion protein expression and for nickel binding isolation of the recombinant protein using a Maxwell 16™ protein isolation robot (Promega Inc, Madison, WI, USA). Panel B is an SDS polyacrylamide gel stained with Commasie blue, Lane 1: Molecular Weight Marker Proteins (BIO-RAD, Hercules, CA); Lane 2: pRSET-CTB protein (16.1kDa); Lane 3: the CTB-INS fusion protein isolated and purified from BL21 E. coli cells containing pRSET-CTB-INS (23.4kDa).
with fresh medium. The cell cultures were kept in their original plates until harvested to avoid induction of DC maturation caused by mechanical stress associated with replating the cells. The percentage of monocyte-derived immature dendritic cells (M-iDC) was determined directly by flow cytometry based on expression of DC specific cell surface markers (CD14⁻HLA-DR⁺CD11c⁺) after 6 days further incubation.

**DC Maturation Assay and Phenotyping**

The iDCs were stimulated by the addition of CTB (10ug/ml), insulin (10ug/ml), CTB-INS fusion protein (20ug/ml) and Phorbol myristate acetate (PMA) and Ionomycin at 10ng/ml (PMA+Ionomycin) for 48 hrs at 37°C in 5% CO₂. After incubation, the expression of DC surface markers indicating their state of activation and maturation (CD14, CD11c, and HLA-DR) was determined by flow cytometry. Briefly, the gated CD14⁻HLA-DR⁺CD11c⁺ cell population was analyzed for the expression of CD86, and CD83 activation markers (BD Pharmingen, San Jose, CA USA).

**Flow Cytometry Analysis**

For flow cytometry examination of DC surface markers, the DCs were surface stained with antibodies conjugated to Phycoerythrin (PE), Fluorescein isothiocyanate (FITC), Allophycocyanin (APC), and Peridinin Chlorophyll Protein Complex (PerCP) (Parrish, Baez et al. 2009) and resuspended in 1.0% of freshly prepared formaldehyde for analysis using the FACSCalibur flow cytometer. The following antibodies were used to identify the different cell subsets: anti-CD86-FITC, anti-CD83-PE, anti- HLA-DR PerCP anti-CD14-APC anti-mouse IgG1 FITC, anti-mouse IgG1 PE, anti-mouse IgG1 HLA-DR,
anti-mouse IgG1 APC (BD Pharmingen); 7-Amino-actinomycin D (7-AAD) was used to
assess viability of the collected cells. Briefly, a pooled sample of harvested cells was
incubated with 7-Amino-actinomycin D (7-AAD) and prepared for flow cytometry
according to the manufacturer’s instructions (eBioscience, San Diego, CA, USA).
Fluorescence intensity on all flow histograms is shown on a log scale. Forward scatter
(FSC) and Side Scatter (SSC) and percentage of cell population on histograms are shown
in a linear scale.

Secreted Cytokine Analysis by Cytometric Bead Array

Immature DCs were stimulated with PMA+Ionomycin and proteins for 48 hrs as
described above. The supernatant was collected and stored at -20°C until analyzed for
concentrations of IL-10 and IL-12 using the cytometric Bead Array (CBA) kit (Becton
Dickinson Biosciences, San Jose, CA, USA). Briefly, 50 μl of premixed beads coated
with capture antibodies was incubated with 50μl of cytokine standards or test samples in
the dark at room temperature for 1 hr. Following the incubation, 50μl of a mixture of PE-
conjugated antibodies prepared against the specific cytokines was added to each
preparation, and the samples incubated for 2 hrs in the dark at room temperature. The
beads were washed once with the wash buffer supplied with the CBA kit and analyzed
immediately in a BD FACSCalibur flow cytometer (Becton, Dickinson Inc.). Data
analysis was performed using the FCAP Array software packages supplied by BD
Biosciences. Three thousand counting events were acquired from each sample. Cytokine
calibration curves were generated using cytokine standards supplied by BD Biosciences
(Potapova, Gaudette et al. 2007; LaFrance, Kehinde et al. 2008).
Results

CTB-INS Mediated Suppression of DC Maturation involves
Upregulation of TLR2

To determine whether TLR2 or TLR4 was involved in CTB-INS mediated DC activation, flow cytometric analysis of TLR2 and TLR4 markers was carried out on vaccine inoculated activated DCs. The results of flow cytometry analysis indicated that CTB and CTB-INS inoculated DCs significantly upregulated the presence of TLR2 proteins on the surface of the vaccine inoculated DC membrane in comparison with unstimulated DCs, insulin treated DCs and PMA+Ionomycin treated DCs (p<0.05) (Fig. 2.2). No significant up-regulation of TLR4 receptor protein was detected on the surface of CTB, CTB-INS and insulin treated DCs. However, TLR4 was significantly upregulated in PMA+Ionomycin treated cells compared to CTB and CTB-INS treated cells (p<0.05), a result consistent with those obtained earlier by Thierry et al. (2005) (Roger, Miconnet et al. 2005). The finding that TLR2 is upregulated by CTB-INS treated cells and not on insulin treated cells suggests that CTB-INS mediated suppression of DC maturation, similar to that found for the *E. coli* heat sensitive enterotoxin B subunit is attributable to CTB activation of iDCs through induction of TLR2 rather than TLR4.
Figure 2.2: TLR2 upregulation by CTB-INS. The bar graph represents the percentages of dendritic cells expressing Toll-like receptors 2 and 4 (TLR2 and TLR4). Umbilical cord blood monocyte derived immature DC were stimulated for 48 hrs with CTB, CTB-INS, Insulin and PMA+Ionomycin and the induction of TLR2(CD282) and TLR4(CD284) was determined by flow cytometric TLR-specific antibody surface staining methods. The mean and standard error of the mean (SE) were calculated for three separate experiments (p<0.05).
CTB-INS Treatment Suppresses Maturation of Dendritic Cells

The influence of CTB, Insulin, CTB-INS and PMA+Ionomycin on immature DC (iDC) activation and maturation was examined by measuring the expression of DC synthesized co-stimulatory molecules CD86 and CD83. iDC were differentiated from monocytes as described in methods and materials section and assessed for the expression of CD14-HLA-DR+CD11C+ surface markers indicating DC differentiation from monocytes (Fig. 2.3A). The iDC (CD14-HLA-DR+CD11C+) were inoculated with CTB, INS and CTB-INS fusion proteins and assessed for stimulation of DC membrane surface marker expression, indicating activation and maturation, i.e., CD86 and CD83, respectively (Fig. 2.3B). Flow cytometric data obtained for CTB-INS treated cells revealed that iDC exposure to CTB-INS resulted in a significant level of suppression of CD86 and CD83 costimulatory factors as compared with CTB, INS, and PMA+Ionomycin (p<0.05). These results suggest that the physical linkage of CTB to the Insulin autoantigen is responsible for suppression of DC activation and maturation by CTB-INS.
Figure 2.3 Dendritic Cell Activation and Maturation is Suppressed by CTB-INS Fusion Protein. Panel A: Flow cytometry dot plot showing dendritic cells differentiated from monocytes isolated by ficoll gradient centrifugation from human umbilical cord blood. The CD14+ monocytes were supplemented with granulocyte macrophage colony stimulating factor (GM-CSF) and IL-4 and were cultured for 6 days to obtain CD14-HLA-DR+CD11c+ immature human DCs. To determine the viability of DCs isolated from the entire population of collected cells (Upper left panel), 7-AAD negative cells were gated (Upper right panel) and analyzed for expression of the surface marker CD14 (Lower left panel). The CD14- cells were gated and analyzed for co-expression of HLA-DR and CD11c differentiation markers by flow cytometry (Lower right panel). Panel B: The histograms depict the expression of CD83 and CD86 after stimulation of DCs for 48 hrs with CTB, CTB-INS, Insulin and PMA+Ionomycin. Maturation state of the DCs was determined by flow cytometric analysis. The data are representative of three independent experiments with comparable results.
Linkage of CTB to Autoantigen is Necessary for Suppression of DC Activation

Based on the results obtained from the insulin stimulation studies described above, it was important to examine the role of CTB in the observed suppression of DC activation. Therefore immature DCs were stimulated with 10ug insulin alone, co-delivered with 10ug CTB and finally stimulated with equivalent amounts of CTB-INS fusion protein. Co-delivery of insulin + CTB resulted in significant CD83 and CD86 co-stimulatory factor upregulation on DC compared to unstimulated DCs (p<0.05). However, cells treated with CTB-INS fusion proteins showed almost no CD83 and CD86 upregulation as measured by mean fluorescent intensity analysis for each treatment group (Fig. 2.4A). Samples treated with CTB, CTB-INS and PMA+Ionomycin showed a significant increase in cell populations expressing CD86 and CD83 in comparison with the control (unstimulated) DC sample (p<0.05). Interestingly, the population of cells expressing CD83 was significantly reduced in comparison with the population of cells expressing the CD86 costimulatory factor (p<0.05). However the ratios of costimulatory expression for each treatment group remained unchanged (data not shown). Taken together, the data indicate that the physical linkage of CTB to the autoantigen is essential for achieving high levels of immunological suppression of DC activation.
A.

CD 86

- PMA+ IONOMYCIN
- INSULIN +CTB
- CTB-INS
- UNSTIMULATED

% of Max

CD 86 Fluorescence Intensity

B.

CD 83

- PMA+ IONOMYCIN
- INSULIN +CTB
- CTB-INS
- UNSTIMULATED

% of Max

CD 83 Fluorescence Intensity
Figure 2.4: Effects of CTB on Insulin Mediated DC Activation

Immature monocyte derived DCs were stimulated for 48 hrs with 10ug insulin + 10ug CTB, 20ug CTB-INS and PMA+Ionomycin as a positive control for DC activation. The activation and maturation of DCs measured by expression of CD86 and CD83 cell surface markers was determined by flow cytometric methods. The mean fluorescence intensity (MFI) and percentage population data is representative of four repeated independent experiments with comparable results. Panel A: The overlapping histograms depict the expression of CD83 and CD86 costimulatory factors in immature dendritic cells after stimulation with PMA+Ionomycin, Insulin + CTB and CTB-INS. The statistical significance for difference in MFI between treatment groups was calculated based on p<0.05.
Increased Amounts of Insulin Induce Dendritic Cell Activation

The effect of increasing concentrations of insulin protein on DC activation was investigated by stimulating iDCs with increasing amounts of insulin from 10ug/ml up to 250ug/ml. There was no significant difference in the upregulation of CD86 costimulatory molecules on the DC cell surface for concentration 10ug/ml up to 50ug insulin/ml. However, mean fluorescence intensity measurements suggest a progressive increase in the expression of CD86, when the concentration of insulin was increased to 100ug/ml and 250ug/ml respectively (Fig. 2.5A). We also observed an increase in the percentage of the DC cell population activated by increasing the amount of insulin P<0.05 (Fig. 2.5B). Based on the increasing levels of CD86 fluorescence intensity detected, increases in the percentage of activated DCs were shown to correlate with increasing amounts of insulin. Our experimental data did not reveal a significantly different level of CD83 protein synthesized in cells incubated with 100ug/ml and 250ug/ml insulin. Predictably, PMA+Ionomycin induced significantly increased levels of DC synthesis of CD86 and CD83 costimulatory factors compared to other treatment groups (p<0.05).
A.

CD 86

Mean Fluorescence Intensity

0 20 40 60 80 100 120
NO stimulation 10ug Insulin 50ug Insulin 100ug Insulin 250ug Insulin PMA+ Ionomycin

B.
Figure 2.5: Increasing Insulin Dosage Results in DC Activation. Immature monocyte derived DCs were stimulated for 48 hours with different amounts of insulin and PMA+Ionomycin. Activation of DCs was determined by flow cytometric methods. The data presented are representative of more than three independent experiments with comparable results. Panel A: Graph indicating increasing mean fluorescence intensity of CD86 expression on DC cell surfaces with increasing amounts of insulin. Panel B: Histogram showing the percentages of cells activated by different concentrations of insulin as measured by CD86 costimulatory factor expression on the DC cell surface. *p<0.05 for statistical differences between treatment groups.
CTB-INS Stimulates Increased Synthesis of IL-10

To determine whether CTB-INS suppression of DC maturation (suppression of co-stimulatory factor synthesis), resulted in increased secretion of immunosuppressive cytokine IL-10 and decreased synthesis of IL-12, the supernatant medium was collected from DCs exposed to 48 hrs incubation with CTB-INS fusion protein and analyzed by cytometric bead assay (CBA) based flow cytometry for the presence of secreted cytokines. The results of this experiment showed that there was a significant increase in IL-10 production by DCs incubated with CTB-INS compared to DCs stimulated with the autoantigen alone (p<0.05) with a concurrent decrease in proinflammatory cytokine IL-12/23p40 oligomer compared to PMA+Ionomycin treated DCs (p<0.05). Incubation of immature DCs with CTB-INS resulted in a 2.5-fold pg/ml increase in synthesis of IL-10 in comparison with DC incubation with CTB, insulin or PMA+Ionomycin treated cells. Although CTB alone appeared to stimulate DC IL-10 synthesis, the cytokine levels were significantly lower than CTB-INS induced DC IL-10 production (p<0.05) (Fig. 2.6). In summary, CTB-INS induced suppression of DC activation and maturation appears to be followed by an increased secretion of the anti-inflammatory cytokine IL-10 and a decrease in secreted IL-12/23p40, suggesting the possibility of immune suppression of inflammatory cytokine synthesis.
Figure 2.6. CTB-INS Fusion Protein Stimulates IL-10 Synthesis
Panel A: The histograms show the mean fluorescence intensity of IL-10. Premixed plastic beads coated with capture antibodies (BD Biosciences, San Jose, CA, USA) and a mixture of Phycoerythrin-conjugated antibodies against IL-10 were incubated for 2 hrs with the supernatant removed from different treatment conditions of proteins with DCs as described in the Materials and Methods section. The beads were washed and analyzed by flow cytometry for determination of the fluorescence intensity of bound IL-10. Panel B: Cytometric Bead Array-defined concentrations of IL-10 and IL-12/23p40 subunit measured from cell supernatants taken from DCs receiving different treatment conditions. For each treatment the concentration of IL-10 and IL-12/23p40 subunit was normalized to standard IL-10 and IL-12/23p40 cytokine curve and given in pg/ml. The data are the Mean and SE (*P<0.05) showing statistically significant differences between CTB-INS treated cells and PMA + Ionomycin treated cells for repeated independent experiments.


**Discussion**

Our laboratory and others previously demonstrated the phenomenon of suppression of insulitis and autoimmune diabetes in NOD mice inoculated with CTB-INS (Bergerot, Ploix et al. 1997; Arakawa, Yu et al. 1998; Sobel, Yankelevich et al. 1998). However, the cellular and molecular mechanisms underlying CTB-proinsulin – immune cell interactions involved in the suppression of disease pathology are incompletely understood. Because DCs are the dominant population of APCs involved in the induction of inflammatory and autoreactive T cell morphogenesis, we investigated early effects of CTB-INS on human iDC activation and maturation *in vitro* and attempted to correlate these results with findings from NOD mice diabetes onset studies.

Our experiments suggest that CTB when conjugated to pro-insulin (CTB-INS) results in the suppression of DC activation and maturation as determined by a failure of CTB-INS and to a limited extent proinsulin alone to stimulate the upregulation of CD86 and CD83 costimulatory factors on iDC. This observation was further confirmed when CTB-INS stimulated DCs resulted in an increase in DC secretion of the anti-inflammatory cytokine IL-10. Further, our experimental data appears to confirm previous studies with the heat sensitive enterotoxin from *E. coli* (LTB), that CTB, a molecule similar in structure to LTB like LTB selectively stimulates TLR2 activation in response to DC incubation with CTB or CTB-INS proteins. Previous observations show that CTB can greatly enhance the immunogenicity of linked antigens (George-Chandy, Eriksson et al. 2001; D'Ambrosio, Colucci et al. 2008) by inducing DC upregulation of CD86 and CD83 (George-Chandy, Eriksson et al. 2001; Isomura, Yasuda et al. 2005). Morphological changes which included enlargement of iDC, elongation of DC dendrites
and increased migration of DCs into draining lymph nodes observed in those studies was not monitored in the present study. D’Ambrosio et al. suggested that CTB could partially prevent LPS-induced maturation of monocyte-derived DCs while Morita et al. reported that CTB induced DC maturation. Careful consideration of these findings appears to suggest that CTB may play a dual role as both an immunostimulatory and immunosuppressive modulator molecule. We report that CTB activation of DCs appears to be less than PMA+Ionomycin induced activation of DC maturation. However, when compared to insulin and CTB-INS, CTB shows a significantly increased level of DC activation. In essence, the question of the duality of CTB activity either as a pro-inflammatory or anti-inflammatory immune response modulator may be resolved by understanding the activity of CTB in relation to another molecule (antigen or autoantigen).

Earlier NOD mouse insulin inoculation experiments conducted by Bergerot et al., demonstrated sustained prevention of diabetes onset, even when oral insulin treatment was initiated as late as 15 weeks after birth of the mice. In addition, the protective effect against continued insulitis development was transferable to untreated NOD mice through transfer of CD4+ T cells from CTB-INS inoculated animals (Bergerot, Ploix et al. 1997). Our recent finding that CTB-INS is active in the suppression of DC maturation provides a possible explanation for CTB enhanced insulin-mediated diabetes suppression observed in NOD mice studies employing CTB-INS fusion protein produced in viruses, plants, and silkworm larvae (Denes, Krausova et al. 2005; Gong, Jin et al. 2007; Gong, Long et al. 2009).

Previous studies demonstrated that insulin can function as a prime target for
autoantigen directed T1D therapies (Palmer, Asplin et al. 1983; Jaeckel, Klein et al. 2003; Jaeckel, Lipes et al. 2004; Kent, Chen et al. 2005). These studies showed that administration of insulin analogs, such as altered B: 9-23 insulin, induced T cell mechanisms involved in the prevention of diabetes development in NOD mice (Weiner, Zhang et al. 1991; Zhang, Davidson et al. 1991; Elliott, Qin et al. 1994; Atkinson and Leiter 1999; Weiner 2001; Kobayashi, Abiru et al. 2007). However, a major concern is that increasing amounts of insulin can act as a double edged sword with the potential to protect against the development of autoimmunity at low insulin concentrations while stimulating pathogenic immunity at higher insulin concentrations (Romagnani 1998; Kobayashi, Abiru et al. 2007). Liu et al. (2009) recently demonstrated that increased insulin concentration (2.5-25mg/L) significantly increased DC synthesis of co-stimulatory factors, increasing DC ability to activate autologous lymphocytes (Liu, Wang et al. 2009). Our experimental data showed that exposure of iDCs to increasing amounts of insulin resulted in an increase, rather than a further suppression, of DC activation. This result provides a plausible answer to the earlier counter intuitive observation that increased insulin levels might be expected to stimulate rather than suppresses diabetes onset in NOD mice.

The observed increase in suppression of DC maturation by CTB-INS fusion protein in comparison with insulin alone raises the question of whether CTB-INS suppression of DC maturation is mediated through the activation of Toll-like receptor signaling processes. The type II heat-labile enterotoxin (LT-IIIB) of E. coli, which has an AB5 type structure similar to CTB, was shown to induce DC activation through TLR2 resulting in downstream activation of NF-κB (Connell, Metzger et al. 1995; Lencer,
Examination of CTB-mediated suppression of DC activation and maturation similarly suggests that TLR2 may be selectively upregulated as well in CTB stimulated DCs. The CTB subunit may be selective for TLR2 since TLR2 depends specifically on hydrophobic interactions for ligand binding (Okusawa, Fujita et al. 2004; Seong and Matzinger 2004) as observed for LTBIIb. While the specific mechanism by which CTB may interact with TLR2 is largely inferred from LTB studies, further insights may be provided from the binding properties of CTB and other pentameric enterotoxin B subunits that have the capacity to participate in both hydrophobic and hydrophilic interactions (van den Akker, Sarfaty et al. 1996; Tinker, Erbe et al. 2003).

DC activation correlates with expression of the major immunosuppressive cytokine, IL-10, identified in APCs including DC subsets (de Waal Malefyt, Abrams et al. 1991; Sica, Saccani et al. 2000). Autocrine production of IL-10 by iDCs was shown to inhibit synthesis and release of pro-inflammatory cytokines and other molecules (IL-12, TNF-α, IL-6, LTB4, NO, PGE2). Further, IL-10 secretion by DCs was shown to suppress Th1 lymphocyte activity by enhancing down-regulation of costimulatory molecule expression on the DC surface (Grutz 2005; Harizi and Gualde 2006). In the case of experimental encephalitis, oral administration of CTB linked to myelin basic protein (MBP) increased suppression of encephalitis induced by TGF-β originating from CD4 T cells in the animal spinal cord (Sun, Xiao et al. 2000). In the same way, immunological analyses of patients with Behcet’s disease treated with CTB conjugated to HSP60 p336-351 in a pilot clinical trial showed the presence of increased synthesis of IL-10 by CD4+ cells as well as a reduction in T cell synthesis of pro-inflammatory cytokines IL-2 and
IFN-γ (Stanford, Whittall et al. 2004). Through inhibition of the above endogenous pro-inflammatory mediators, IL-10 was shown to be central to maintenance of DCs in an immature state and in the down-regulation of DC-mediated inflammatory responses (Harizi and Gualde 2006). In paracrine fashion, IL-10 synthesized by DCs can regulate immunity by altering the function of different adjacent cell types. In several studies, IL-10 secreted by iDCs stimulated the development of cognate naïve Th0 cells into either Th2 lymphocytes, or suppressor regulatory T cells (Romagnani 1998; Romagnani, Kapsenberg et al. 1998; Stassen, Fondel et al. 2004; Stassen, Schmitt et al. 2004). Studies in knockout mice showed that IL-10 can down-regulate autoimmunity in the intestine and can be active in the suppression of Crohn’s disease (Grimbaldeston, Nakae et al. 2007; Minderhoud, Samsom et al. 2007).

In our experiments, CTB-INS stimulated iDCs produced significantly higher levels of IL-10 than in CTB or proinsulin stimulated iDCs. However of interest, the biosynthesis of IL-12/23p40 subunit was significantly inhibited. This result is critical because induction of cognate naïve T helper cell (Th0) morphogenesis into Th1 effector cells that secrete IFN-γ and IL-2 responsible for islet inflammation and β-cell death is dependent on DC synthesis of IL-12 in addition to expression of surface costimulatory molecules (Pulendran, Smith et al. 1999; Itano, McSorley et al. 2003; Kang and Kim 2006; Zorena, Mysliwksa et al. 2008). Secretion of IL-10 was shown to be essential for the inhibition of DC maturation through its effect of blocking IL-12 synthesis. As a result of increased DC biosynthesis of IL-10, inflammatory autoreactive effector Th1 cell proliferation and secretion of downstream inflammatory cytokines IFN-γ and IL-2 is inhibited. This finding in combination with our experiments demonstrating CTB-INS
inhibition of DC co-stimulatory factor upregulation and IL-12/23p40 synthesis may be critical to determine the probable cellular mechanisms underlying CTB-INS mediated immune suppression of T1D. Future experiments incubating CTB linked autoantigens with naïve DCs will reveal the capacity of CTB-INS to arrest the progression of T1D once hyperglycemia has become established. Establishment of CTB-INS mediated immunological suppression of T1D progression will help to determine whether this form of interventional therapy in combination with anti-inflammatory cytokines can prevent both the onset and the progression of diabetes once hyperglycemia has developed. Once durable immunological suppression of diabetes progression has been achieved, interventional therapy with insulin producing mesenchymal stem cells may provide an effective, safe and durable cure for the present ravages of autoimmune diabetes.

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References


CHAPTER THREE

CHOLERA TOXIN B SUBUNIT LINKED TO GLUTAMIC ACID DECARBOXYLASE SUPPRESSES DENDRITIC CELL MATURATION AND FUNCTION

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List of Abbreviations: DC: dendritic cell; TLR: Toll-like receptor; T1D: type 1 diabetes
PMA: Phorbol myristate acetate; LPS: lipopolysaccharide
Abstract

Dendritic cells (DC), the largest population of antigen presenting cells, regulate the delicate balance between immune responses and tolerance involved in the onset of type I diabetes (T1D). Cholera toxin B subunit (CTB) is a known mucosal adjuvant for the generation of immune responses to linked antigens. However, CTB linked to autoantigens may induce tolerance and therefore suppress the induction of autoimmunity. In this present study, we examined the possibility that CTB linked to a 5 kDa C-terminal protein fragment of the diabetes autoantigen glutamic acid decarboxylase (GAD), may directly suppress the maturation of DCs assessed by CD86, CD83, CD80 and CD40 biosynthesis. Furthermore, we examined the possibility that CTB-GAD\textsubscript{35} may suppress human DC maturation and function in response to phorbol myristate acetate (PMA) and Ionomycin stimulation. Incubation of human umbilical cord blood monocyte-derived DCs (iDCs) with GAD induced low level surface expression of CD86, CD83, CD80 and CD40 co-stimulatory factors compared to untreated samples, while incubation of iDCs with CTB-GAD\textsubscript{35} did not induce expression of CD86, CD83, CD80 and CD40 co-stimulatory factors. Also, our findings showed that CTB-GAD\textsubscript{35} abrogates PMA and Ionomycin induced iDC maturation. Further, suppression of iDC maturation by CTB-GAD\textsubscript{35} is accompanied by a dramatic decrease in the secretion of IL-12/23p40 and IL-6 pro-inflammatory cytokines while increasing the secretion of the immunosuppressive cytokine IL-10. These findings suggest that linkage of CTB adjuvant to the dominant T1D autoantigen GAD may play a critical role in stimulating DC priming of naïve Th0 cell development into immunosuppressive T lymphocytes. This could be important in adjuvant stimulated vaccine responses for suppression of T1D autoimmunity.
Introduction

TYPE 1 DIABETES MELLITUS (T1DM) is an organ-specific autoimmune disease which results from autoreactive lymphocyte destruction of insulin-producing pancreatic islet beta cells (Eisenbarth 1986; Tisch and McDevitt 1996). The loss of islet beta-cell function leads to insulin deficiency and elevated blood sugar levels (hyperglycemia). Hyperglycemia increases levels of cellular oxidative stress leading to chronic inflammation throughout the body. Therefore, diabetics have an increased and premature risk for secondary neural and circulatory health problems, including blindness, heart attack, and stroke (Libby, Nathan et al. 2005).

Dendritic cells (DCs), professional antigen presenting cells (APC), are implicated in mediating islet beta-cell destruction in T1D. Through antigen presentation, DCs play a key role in the polarization and development of naive T helper cells (Th0) into autoreactive Th1 lymphocytes or immunosuppressive Th2 cells. This process is critical for maintenance of immunological homeostasis (Pulendran, Smith et al. 1999; Pulendran, Kumar et al. 2001; Itano, McSorley et al. 2003). Maturation of DCs in response to “self” antigens such as pancreatic islet beta-cell proteins induces the development of autoreactive CD8+ cytotoxic T lymphocytes (CTL) and CD4+ T helper (Th1) lymphocytes in addition to initiating B cell production of autoantibodies. Subsequently, autoreactive Th1 cells infiltrate the pancreatic islets in non-obese diabetic (NOD) mice while secreting inflammatory cytokines such as IFN-gamma and IL-2. These cytokines are known to stimulate macrophage and CTL secretion of oxidative compounds NO, O_{2}, H_{2}O_{2} in addition to other inflammatory cytokines (IL-1 beta, TNF-alpha, TNF-beta) [5-7]. Consequently, these immune responses induce chronic pancreatic inflammation.
(insulitis), which results in apoptosis of the islet β-cells ultimately leading to insufficient levels of insulin production and availability (Piccinni, Beloni et al. 1998).

In addition to induction of conventional Th1 and Th2 cells, DCs induce the development of adaptive regulatory T cells (iTregs) for the maintenance of immunologic tolerance (Kapsenberg 2003). Immuno-cytochemical analyses showed that M cells of the intestinal epithelium uptake autoantigens into peripheral DCs via several routes and this may aid in the establishment of immune tolerance (Yoon and Jun 2005). Following activation, DCs migrate to adjacent lymph nodes. Here, they present antigen peptides on major histocompatibility complex class II (MHCII) receptors, synthesize costimulatory factors, and secrete cytokines. Together, these three signals guide the development of naïve cognate T cells into antigen-specific inflammatory Th1 lymphocytes or anti-inflammatory Th2 lymphocytes. Immature or peripheral DCs (iDCs), that displayed high levels of costimulatory molecule expression and secreted cytokine IL-12, were found to induce Th1 lymphocyte differentiation; while those that displayed low levels of costimulatory molecule expression and secreted cytokine IL-10 induced the development of Th2 lymphocytes (Rissoan, Soumelis et al. 1999; Liu, MacKenzie-Graham et al. 2001; Holmgren, Adamsson et al. 2005; Li, Zhong et al. 2006).

Oral delivery of autoantigens such as insulin and glutamic acid decarboxylase (GAD) resulted in partial suppression of diabetes symptoms in diabetic animals (Zhang, Davidson et al. 1991; Czerkinsky, Anjuere et al. 1999). However, this form of anti-inflammatory therapy requires repeated autoantigen administration over an extended period of time. Adjuvants were considered to enhance autoantigen therapy to further prevent T1DM onset (Weiner, Zhang et al. 1991; Zhang, Davidson et al. 1991). The
mucosal adjuvant, cholera enterotoxin B subunit (CTB) from *Vibrio cholerae*, was shown to be highly efficient for induction of oral tolerance in T1DM when conjugated with islet autoantigens (Sun, Holmgren et al. 1994; Sun, Rask et al. 1996; Sun, Li et al. 2000; Sun, Xiao et al. 2000). The CTB subunit was shown to bind specifically to GM1-ganglioside, a receptor molecule found in common on the membrane of most types of epidermal cells. This facilitates the entry of the holotoxin into the cell (Eriksson and Holmgren 2002). Thus, CTB is an efficient trans-mucosal carrier molecule for autoantigen induction of peripheral tolerance (Sun, Holmgren et al. 1994; Shreedhar, Kelsall et al. 2003). Our laboratory has compared the protective effects of diabetes-specific autoantigens, such as GAD and proinsulin (INS), when genetically conjugated to adjuvants such as heat labile enterotoxin-LTB from enterotoxigenic *Escherichia coli*, the enterotoxin B chain from *Shigella*-STB and the plant toxin ricin B subunit-RTB on diabetes onset in NOD mice. (Carter, Yu et al. 2006).

Our laboratory has recently shown that a possible mechanism for the suppression of T1D onset is the induction of immune tolerance through DC interaction with CTB-autoantigen. The inoculation of human DCs with CTB-INS fusion protein resulted in an enhanced suppression of DC maturation. In addition, CTB-INS stimulated DCs increased their secretion of immunosuppressive cytokine IL-10 with a concurrent suppression of inflammatory cytokine IL-12/23p40 (Odumosu, Payne et al. 2010). We hypothesized that if the mechanism of immune suppression was similar that the fusion protein CTB-GAD$_{35}$ could also exert an inhibitory affect on DC maturation. Thus, through analysis of CTB-GAD$_{35}$ fusion protein interactions with iDCs, we anticipated that we might be able to gain a clearer understanding of the mechanisms underlying CTB-autoantigen fusion protein
interactions with DCs to suppress the onset of T1D.

**Materials and Methods**

Expression and Purification of CTB-GAD\textsubscript{35} Fusion Proteins in E. coli

A cDNA fragment encoding a 5-kDa peptide (amino acid 509-543) proximal to the carboxyl-terminus of GAD\textsubscript{65}—designated GAD\textsubscript{35} was genetically linked to the carboxyl-terminus (309bp) of CTB to generate the fusion gene CTB-GAD\textsubscript{35}. The cholera toxin B subunit–autoantigen fusion gene CTB-GAD\textsubscript{35} was cloned into the (A) configuration of the *E. coli* expression vector pRSET (Invitrogen™, Carlsbad, CA), under control of the bacteriophage T7 promoter in order to achieve high levels of transgene expression. The pRSET vector also contained an oligonucleotide encoding 6 histidines immediately upstream of the CTB permitting isolation of the transgene product. Selective clones were assessed by DNA sequence analysis to confirm the in-frame linkage of CTB and CTB-GAD\textsubscript{35} DNA fragments. Recombinant vectors were then introduced into *E. coli* producer strain BL21 (DE3)pLysS (Invitrogen, Carlsbad, CA) by electroporation for nickel affinity column isolation of the recombinant protein (Carter, Yu et al. 2006).

Recombinant CTB-GAD\textsubscript{35} fusion protein expressed in *E. coli* was purified by isolating the 6xHis-tagged protein from lysed cells using metal chelation chromatography. Transformed *E. coli* strain BL-21, was grown in 250 ml Luria Broth (LB) medium containing ampicillin (100mg/ml) with shaking at 37°C. While still in log phase of growth, CTB-GAD\textsubscript{35} protein synthesis was stimulated by addition of 90 mg isopropyl β-D-1thiogalacto-pyranoside (IPTG), (Sigma Chemical Co. St. Louis, MO), to the bacterial culture. After 6 hr continued growth at 37°C, the bacterial culture was
pelleted by centrifugation in a SA-600 rotor for 10 min, at 5,000 rpm and 4°C, in a Sorvall RC5B centrifuge. The cell pellet was resuspended in 1.0 ml/tube of 10 mM HEPES buffer (pH 7.5), containing 100 mM imidazole. The cells were disrupted by sonication at 3 x 10 sec bursts at 10 W, with a Sonic 60 Dismembrator (Fisher Sci. Sunnyvale, CA). The CTB-GAD$_{35}$ protein was isolated and purified from the bacterial homogenate using a Maxwell Model 16 robotic protein purification system (Promega Inc.™), according to the protein isolation protocol provided by the manufacturer (Promega Inc., Madison, WI). In order to obtain a pure protein product, the robot employs electromagnetically charged Magne-His Nickel-Iron alloy particles with an affinity for the 6-HIS tag linked to the N terminus of the recombinant CTB-GAD$_{35}$ fusion protein. Imidazole was removed from the protein mixture by dialysis of the preparation against 2 x 1.0 Liter, 10 mM HEPES buffer (pH 7.5), for 4 hr at 4°C. The purity of the isolated CTB-GAD$_{35}$ protein (~22kDa) was determined by electrophoretic mobility analysis in a 12% polyacrylamide gel in comparison with protein molecular weight standards (Figure 1b). The purified CTB-GAD$_{35}$ protein was confirmed by immunoblot analysis using an anti-polyhistidine primary antibody and stored at -20°C until further use.

Isolation and Culture of Monocyte-derived DC from Human Cord Blood

Monocyte-derived dendritic cells (MDDC) were prepared from freshly collected human umbilical cord blood. The leukocyte fraction of cord blood obtained from normal healthy placenta donors (by LLU IRB approved protocols) was separated from red blood cell and platelet fractions by Ficoll-paque (GE Healthcare, Uppsala, Sweden) density gradient centrifugation for 30 min at 2,000 rpm@ 4°C, in a Beckman Coulter Allegra X-
15R centrifuge, equipped with a SX4750 rotor. The CD14+ monocytes were obtained from the total lymphocyte fraction by incubation with anti-CD14 PE (Phycoerythrin) (Miltenyi Biotech, Auburn, CA) for 10 min @ 4°C followed by incubation with anti-PE magnetic microbeads for 15 min at 4°C. The cells were separated magnetically by passing them through the MACS column as described by the manufacturer (Miltenyi Biotech) (Devaraj, Glaser et al. 2006; Devaraj, Dasu et al. 2008). The purity of the monocyte fraction was determined by flow cytometry in a BD FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA).

The isolated monocytes were cultured in 6 cm non-pyrogenic polystyrene culture plates in RPMI 1640 culture medium (Mediatech Inc. Manassas, VA, in a humidified atmosphere of 5% CO2 at 37 °C (Preprotech, Rocky Hill, NJ). The medium was supplemented with 10% fetal bovine serum (FBS), 1 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 50 ng/ml human recombinant GM-CSF, and 10 ng/ml human recombinant IL-4 (ProSpec-Tany TechnoGene, Rehovot Science Park, Israel). The monocyte cell culture was fed every 2 days by gentle replacement of 50% of the medium with fresh medium. The cell cultures were kept in their original plates until harvested to avoid induction of DC maturation caused by mechanical stress associated with replating the cells. The percentage of monocyte-derived iDC (M-iDC) was determined directly by flow cytometry based on expression of DC-specific cell surface markers (CD14−HLA-DR+CD11c+) after 6 days further incubation.

DC Maturation Assay and Phenotyping

The iDCs were stimulated by the addition of CTB (10ug/ml), GAD35 (10ug/ml), CTB-
GAD$_{35}$ fusion protein (20ug/ml) and Phorbol myristate acetate (PMA) and Ionomycin at 10ng/ml (PMA+Ionomycin) for 48 hrs at 37°C, in 5% CO$_2$. After incubation, the expression of DC surface markers, indicating their state of activation and maturation (CD14, CD11c, HLA-DR), was determined by flow cytometry. The gated CD14$^-$ HLA-DR$^+$ CD11c$^+$ cell population was analyzed for the expression of CD86, CD83, CD80, and CD40 activation markers (BD Pharnigen, San Jose, CA USA).

Flow Cytometry Analysis

For flow cytometry examination of DC surface markers, the DCs were surface stained with antibodies conjugated to PE, Fluorescein isothiocyanate (FITC), Allophycocyanin (APC), and Peridinin Chlorophyll Protein Complex (PerCP) (Parrish, Baez et al. 2009) and resuspended in 1.0% of paraformaldehyde for analysis using the FACSCalibur flow cytometer. The following antibodies were used to identify the different cell subsets: anti-CD86-FITC, anti-CD83-PE, anti-CD40-FITC, anti-CD80-PE, anti-HLA-DR-PerCP anti-CD14-APC anti-mouse IgG1-FITC, anti-mouse IgG1-PE, anti-mouse IgG1-HLA-DR, anti-mouse IgG1-APC (BD Pharnigen); 7-Amino-actinomycin D (7-AAD) was used to assess viability of the collected cells. Briefly, a pooled sample of harvested cells was incubated with 7-AAD and prepared for flow cytometry according to the manufacturer’s instructions (eBioscience, San Diego, CA, USA). Fluorescence intensity on all flow histograms is shown on a log scale.

Secreted Cytokine Analysis by Cytometric Bead Array

Immature DCs were stimulated with PMA+Ionomycin and proteins for 48 hrs as
described above. The supernatant was collected and stored at -20°C until analyzed for concentrations of IL-6, IL-10 and IL-12/23p40 using the cytometric Bead Array (CBA) kit (Becton Dickinson Biosciences, San Jose, CA). Fifty µl of premixed beads coated with capture antibodies was incubated with 50µl of cytokine standards or test samples in the dark at room temperature for 1 hr. Following the incubation, 50µl of a mixture of Phycoerythrin-conjugated antibodies prepared against the specific cytokines was added to each preparation, and the samples were incubated for 2 hrs in the dark at room temperature. The beads were washed once with the wash buffer supplied with the CBA kit and analyzed immediately in a BD FACSCalibur flow cytometer (Becton, Dickinson Inc.). Data analysis was performed using the FCAP Array software packages supplied by BD Biosciences. Three thousand counting events were acquired from each sample. Cytokine calibration curves were generated using cytokine standards supplied by BD Biosciences (Potapova, Gaudette et al. 2007; LaFrance, Kehinde et al. 2008).

**Statistical Analysis**

Statistical analyses of experimental treatment groups were performed using a one-way analysis of variance method (ANOVA). Cell population percentages and fluorescence intensity values were presented as the mean ± 95% confidence interval. A $p< 0.05$ was considered statistically significant.

**Results**

CTB-GAD$_{35}$ Fusion Protein Expression in *E.coli*

Recombinant GAD$_{35}$ and CTB-GAD$_{35}$ were identified by immunoblot with a
monoclonal antibody made against the carboxyl terminal epitope of GAD$_{65}$. The CTB-GAD$_{35}$ protein was identified in both monomeric (~22kDa) and multimeric form (Fig. 3.1A). In addition, identification of GAD$_{35}$ and CTB-GAD$_{35}$ was confirmed by immunoblot analysis using an anti-polyhistidine antibody.
3.1A.

3.1B.

Arrow = CTB pentamer
Figure 3.1. A. Diagram of E. coli pRSET (A) expression vector. The cDNA fragment of cholera toxin B subunit (CTB) C-terminus was fused to the 5kDa pancreatic islet autoantigen glutamic acid decarboxylase (GAD). The vector includes a bacteriophage T7 promoter to generate high expression levels of inserted genes and a 6 histidine sequence tag for nickel affinity column isolation of recombinant protein. (B) Immunoblot identification of renatured GAD$_{35}$ and CTB-GAD$_{35}$ subunit fusion proteins synthesized in E. coli. Proteins identified with anti-His primary antibody.
CTB-GAD$_{35}$ Suppresses Monocyte-derived Dendritic Cell Activation and Maturation

We examined the influence of CTB, GAD$_{35}$, CTB-GAD$_{35}$ and lipopolysaccharide (LPS) on immature DC maturation by measuring the surface expression of costimulatory molecules CD86, CD83, CD80 and CD40. Immature dendritic cells (iDC) were differentiated from monocytes and assessed for the expression of CD14-HLA-DR+CD11c+ surface markers indicating DC differentiation from monocytes. iDCs (CD14-HLA-DR+CD11c+) were inoculated with CTB, GAD$_{35}$ and CTB-GAD$_{35}$ fusion proteins and assessed for surface expression of CD86, CD83, CD80 and CD40 (Fig. 3.2). Flow cytometric data obtained for CTB-GAD$_{35}$ treated cells revealed that iDC exposure to CTB-GAD$_{35}$ resulted in a significantly lower expression of CD86, CD83, CD80 and CD40 costimulatory factors as compared with CTB, GAD$_{35}$, and LPS (p<0.05). These results suggest that the physical linkage of CTB to the GAD$_{35}$ autoantigen is responsible for suppression of dendritic cell activation and maturation by CTB-GAD$_{35}$. 
Figure 3.2. CTB-GAD$_{35}$ suppresses activation and maturation of DCs. Dendritic cells were stimulated for 24 hrs with CTB, GAD$_{35}$, CTB-GAD$_{35}$ and LPS. Markers of DC activation and maturation were determined using flow cytometry. Histograms depict the expression of CD86, CD83, CD80 and CD40 after stimulation. Shaded histogram represents untreated sample (negative control).
CTB-GAD₃₅ Downregulates PMA and Ionomycin-induced DC Surface Costimulatory Molecule Expression

Based on the results obtained showing that CTB-GAD₃₅ suppresses DC maturation, we further assessed the capacity of CTB-GAD₃₅ to prevent activation of DCs in the presence of PMA+Ionomycin stimulation. Separate cultures of DCs were treated with PMA and Ionomycin alone and a combination of PMA+Ionomycin plus CTB-GAD₃₅. In preliminary experiments, 24-hr and 48-hr treatments were tested. Results showed that the effects of CTB-GAD₃₅ on PMA+Ionomycin DCs were more evident during the first 24 hrs (data not shown). Therefore, in subsequent experiments, we used the 24-hr time points. As shown in Figure 3.3, CTB-GAD₃₅ induces a statistically significant reduction of PMA+Ionomycin induced up-regulation of CD86, CD83 and CD80 (p<0.05).
Figure 3.3. CTB-GAD\textsubscript{35} suppresses PMA+Ionomycin induced maturation of DCs. Dendritic cells were stimulated with PMA+Ionomycin and a combination of PMA+Ionomycin and CTB-GAD\textsubscript{35} for 24 hrs. Maturation of DCs was determined using flow cytometry. Histograms depict the expression of CD86, CD83, CD80 DC costimulatory factors following fusion protein, PMA and Ionomycin stimulation.
CTB-GAD$_{35}$ Suppresses DC Pro-inflammatory Cytokine Secretion

In these experiments, we determined whether CTB-GAD$_{35}$ suppression of DC maturation (suppression of costimulatory factor surface expression) resulted in a decreased synthesis of IL-12/23p40 and IL-6, along with an increased secretion of immunosuppressive cytokine IL-10. The culture medium was collected from DCs exposed to 48 hrs incubation with CTB-GAD$_{35}$ fusion protein and analyzed for the presence of secreted cytokines by cytometric bead array based flow cytometry. The results of this experiment showed that there was a significant decrease in pro-inflammatory cytokines IL-12/23p40 and IL-6 with a concomitant increase in IL-10 production by DCs incubated with CTB-GAD$_{35}$ compared to DCs stimulated with the autoantigen alone (P<0.05) (Fig. 3.4). Therefore, taken together, the observed down regulation of co-stimulatory surface marker expression, in addition to the significant decrease in secreted IL-12/23p40 and IL-6 inflammatory cytokines and increased secretion of the anti-inflammatory cytokine IL-10, CTB-GAD$_{35}$ clearly appears to have an important role in the suppression DC activation and maturation.
Figure 3.4: The CTB-GAD$_{35}$ fusion protein suppresses the secretion of proinflammatory cytokines IL-12/23p40 and IL-6 while increasing synthesis of the anti-inflammatory cytokine IL-10. Premixed plastic beads coated with capture antibodies (BD Biosciences, San Jose, CA, USA). And a mixture of Phycoerythrin-conjugated antibodies against IL-12/23p40, IL-6 and IL-10 were incubated for 2 hr with media removed from DC cultures incubated with CTB and GAD autoantigen treatment conditions as described in the section on materials and methods. The beads were washed and analyzed by flow cytometry to determine the concentrations of IL-10, IL-6 and the IL-12/23p40 subunit. The cytokine concentrations in the samples were normalized to a standard IL-10, IL-6 and IL-12/23p40 cytokine curve and concentrations were provided in pg/ml. The data represent the Mean and SE for cytokine concentration in pg/ml (*p < 0.05) of repeated independent CTB-GAD fusion treatment experiments in comparison with the uninoculated cell control sample, autoantigen alone and/or PMA+ Ionomycin treatment samples.
Discussion

Using a CTB-insulin fusion protein, Bergerot et. al. demonstrated sustained prevention of diabetes onset in NOD mice as late as 15 weeks after birth. Further, a sustained protective effect against the development of insulitis was shown to be transferable to un-inoculated NOD mice through adoptive transfer of CD4+ T cells from CTB-INS inoculated animals (Bergerot, Ploix et al. 1997). Additional experiments in our laboratory and by others have demonstrated that inoculation with CTB-autoantigen fusion proteins suppresses insulitis and autoimmune diabetes in NOD mice (Bergerot, Ploix et al. 1997; Arakawa, Yu et al. 1998; Sobel, Yankelevich et al. 1998). Specifically, our animal immunization studies revealed that CTB-GAD\textsubscript{35} inoculated animals showed reduced levels of hyperglycemia and pancreatic islet inflammation as compared to controls (Denes, Krausova et al. 2005; Denes, Yu et al. 2006). In a separate \textit{in vivo} study, our laboratory recently demonstrated that a combinatorial therapy of CTB-GAD\textsubscript{35} + IL-10 is able to completely suppress the onset of diabetes in NOD mice (Denes, Fodor et al. 2010). However, molecular and cellular mechanisms underlying tri-component vaccine suppression of T1D remain to be elucidated. The dominant role of DCs in the induction of inflammation and tolerance implicates them in an early stage of the mechanism involved in CTB-GAD\textsubscript{35} suppression of diabetes onset.

DC synthesis of IL-12 and expression of surface costimulatory molecules are responsible for induction of T cell morphogenesis into pro-inflammatory autoreactive Th1 effector cells that secrete inflammatory cytokines IFN-\(\gamma\) and IL-2. This T cell response is responsible to a large extent for the onset and development of insulitis and \(\beta\)-cell death in T1D (Pulendran, Smith et al. 1999; Itano, McSorley et al. 2003; Kang and
Kim 2006; Zorena, Mysliwska et al. 2008). Conversely, production of IL-10 by iDCs was shown to suppress biosynthesis and secretion of pro-inflammatory cytokines and other molecules (IL-12, TNF-α, IL-6, LTB4, NO, PGE2). Synthesis of IL-10 was also shown to be essential for the inhibition of DC maturation by suppressing IL-12 synthesis. As a result of increased DC IL-10 production, autoreactive Th1 cell proliferation and secretion of downstream inflammatory cytokines IFN-γ and IL-2 is inhibited. Here we report that the adjuvant – autoantigen fusion protein CTB-GAD35 stimulated iDCs to synthesize significantly higher levels of IL-10 than was observed for iDCs stimulated with CTB or PMA+Ionomycin.

Th17 cells have become very important for the treatment of autoimmune diseases because they have been identified as significant effector cell types involved in both human and mouse autoimmune models (Fujino, Andoh et al. 2003; Nakae, Nambu et al. 2003; Komiyama, Nakae et al. 2006). IL-17 was first detected in the serum and tissues of patients with autoimmune diseases such as multiple sclerosis (MS), systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) (Wong, Ho et al. 2000; Tzartos, Friese et al. 2008). In addition, IL-17 blockade was shown to prevent the development of EAE while IL-17 knockout mice showed resistance to the development of EAE and collagen-induced arthritis (CIA) (Nakae, Nambu et al. 2003; Hofstetter, Ibrahim et al. 2005). Together, these findings suggest that the regulation of Th17 differentiation and function may prove to be a potential treatment for autoimmune diseases. IL-6 has been found to be an effective target for down-regulating the differentiation of Th17 cells as a treatment for autoimmune diseases (Nishimoto, Terao et al. 2008; Yokota, Imagawa et al. 2008). This inflammatory cytokine has evolved as a pleiotropic cytokine involved in regulating the
balance of IL-17 producing Th17 cells and Treg cells (Bettelli, Carrier et al. 2006; Mangan, Harrington et al. 2006). In this study, our findings show that CTB-GAD₃₅ suppresses DC synthesis of IL-6. This result in combination with CTB-GAD₃₅ inhibiting DC co-stimulatory factor up-regulation may be critical elements in the elucidation of cellular mechanisms responsible for CTB-GAD₃₅ mediated immune suppression of T1D.

The efficacy of diabetes autoantigens as a vaccine for suppression of autoimmunity appears to be increased when physically conjugated to an adjuvant. The conjugation of CTB to antigens was shown to significantly enhance the immunogenicity of linked antigens (George-Chandy, Eriksson et al. 2001; D'Ambrosio, Colucci et al. 2008) through induction of DC upregulation of CD86 and CD83 (George-Chandy, Eriksson et al. 2001; Isomura, Yasuda et al. 2005). D’Ambrosio et al. showed that CTB could partially prevent LPS-induced maturation of monocyte derived DCs (D'Ambrosio, Colucci et al. 2008). Recently, we reported our that CTB conjugated to the autoantigen proinsulin (CTB-INS) suppressed DC surface expression of CD86 and CD83 costimulatory molecules with a significant increase in the IL-10 synthesis and a suppression of IL-12/23p40 synthesis (Odumosu, Payne et al. 2010). Similar to D’Ambrosio’s findings, our data suggest that CTB influences DC phenotype and function. Furthermore, CTB may possess an intrinsic capacity to modulate either inflammation or tolerance depending on whether it is conjugated to an antigen or an autoantigen. Further studies are necessary to resolve the question of the duality of CTB activity as an adjuvant for inflammatory or anti-inflammatory immune responses.

In this study, we found that CTB-GAD₃₅ suppressed iDCs capacity to mature and become activated in vitro. Furthermore, our data show that the CTB-GAD₃₅ fusion
protein inhibits PMA+Ionomycin induction of DC maturation as determined by a failure to upregulate CD86, CD83 and CD80 co-stimulatory molecules. This effect suggests that inoculation with the fusion protein may inhibit the progression of previously established autoimmunity e.g., in the case of T1DM, blockage of DC maturation could inhibit further development of diabetes symptoms of insulitis and hyperglycemia, a result partially demonstrated in earlier studies from our laboratory (Arakawa, Chong et al. 1998; Arakawa, Yu et al. 1999). Further, our experiments indicated that CTB-GAD35 inoculated DCs were inhibited in their ability to synthesize major pro-inflammatory cytokines IL-12/23p40 and IL-6. This loss was balanced with a concomitant increase in the synthesis of the anti-inflammatory cytokine IL-10. The results of our recent CTB-INS studies establish a molecular basis for CTB-GAD35 suppression of diabetes onset in NOD mice (Denes, Krausova et al. 2005; Gong, Jin et al. 2007; Gong, Long et al. 2009). In earlier studies, we demonstrated that fusion of CTB to the autoantigen was essential for enhancement of immune suppression, as co-delivery of CTB and the autoantigen did not result in a significant inhibition of DC activation and maturation (Odumosu, Payne et al. 2010). Together our experimental findings suggest that linkage of CTB to the autoantigen is key to identification of the mechanism underlying adjuvant-enhanced tolerance. Establishment of whether CTB-GAD35 mediated immunological suppression of DCs can halt or reverse T1D progression will provide the basis for understanding how this form of interventional therapy can be applied to prevent, halt or reverse the onset of insulitis and hyperglycemia in diabetes patients.
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References


CHAPTER FOUR

ADJUVANT-AUTOANTIGEN FUSION PROTEIN INOCULATED HUMAN DENDRITIC CELLS DIRECT ANTI-INFLAMMATORY T CELL MORPHOGENESIS

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List of Abbreviations: DC: dendritic cell; TLR: Toll-like receptor; T1D: type 1 diabetes PMA: Phorbol myristate acetate; LPS: lipopolysaccharide. Th: T helper cell, Treg: regulatory T cell
Abstract

Linkage of the pancreatic islet proteins proinsulin (INS) and glutamic acid decarboxylase (GAD) to the cholera toxin B chain (CTB) enhances autoantigen suppression of type 1 diabetes (T1D). In human tissues the CTB::INS fusion protein was shown to suppress dendritic cell (DC) biosynthesis of costimulatory factors required for the initiation of autoreactive T cell morphogenesis. In this study, we show that CTB::INS/GAD autoantigen inoculated immature DCs guide T cell development in the direction of anti-inflammatory Th2 lymphocytes and regulatory T cells (Tregs). Further, our experimental findings show that CTB-pancreatic autoantigen fusion protein inoculated DCs suppress the proliferation of inflammatory CD4 and CD8 T lymphocytes thought to play a major role in the initiation of diabetes onset. Inoculation of immature DCs with CTB::INS/GAD fusion proteins significantly increased cognate T cell secretion of immunosuppressive cytokines IL-10 and TGF-β. In contrast, fusion protein inoculated DCs suppressed lymphocyte secretion of the pro-inflammatory cytokines IFN-γ, IL-2 and IL-17. Taken together, our experimental data suggests that CTB-autoantigen fusion protein inoculated immature DCs guide initial stages of naïve T cell development in the direction of immunological tolerance while simultaneously suppressing autoreactive T cell development. Together the experimental results provide a plausible mechanism for initiation of CTB-autoantigen mediated immunological suppression of T1D in humans. The absence of DC apoptosis and necrosis during the course of these studies suggests that CTB-autoantigen fusion proteins are an effective and safe vaccination strategy for prevention of diabetes onset in susceptible patients.
**Introduction**

Type 1 Diabetes mellitus (T1D) is a chronic organ-specific autoimmune disease that begins in childhood and persists throughout life resulting in progressive destruction of the pancreatic insulin and glutamic acid decarboxylase-producing islet beta cells (Eisenbarth 1986; Tisch and McDevitt 1996). The continual loss of islet \( \beta \)-cell function leads to insulin deficiency and elevated blood sugar levels (hyperglycemia). The persistence of high blood sugar levels increases cellular oxidative stress leading to chronic inflammation in almost all organs of the body. Thus, the result is an increased risk for a variety of secondary circulatory and neural physiological complications, including blindness, kidney failure, heart attack, and stroke which lead to unacceptable levels of premature mortality (Libby, Nathan et al. 2005).

Previous studies have shown that partial suppression of diabetes symptoms can be achieved by oral delivery of small amounts of the dominant pancreatic islet autoantigens glutamic acid decarboxylase (GAD) and insulin (Zhang, Yuen et al. 1991; Czerkinsky, Anjuere et al. 1999). However, in diabetic animals, earlier studies showed that suppression of insulitis and hyperglycemia could be enhanced through conjugation of islet autoantigens to the non-toxic B subunit of *Vibrio cholerae* enterotoxin (CTB) (Bergerot, Ploix et al. 1997; Arakawa, Chong et al. 1998; Arakawa, Yu et al. 1998; Arakawa, et al. 1999). Further experiments showed that CTB is an efficient transmucosal carrier molecule for autoantigen induction of peripheral tolerance (Sun, Holmgren et al. 1994; Shreedhar, Kelsall et al. 2003). Experiments comparing the efficacy of CTB to the B subunits of several related bacterial enterotoxins revealed that most bacterial and plant enterotoxin B subunits possessed a similar level of efficacy for
enhancement of autoantigen mediated diabetes suppression (Carter, Yu et al. 2006).

Dendritic cells (DCs) are generally thought to be the most potent of the professional antigen presenting cells (APC) implicated in mediating islet β-cell destruction in T1D. Through antigen presentation to cognate T cells, DCs play a key role in the onset and regulation of adaptive immune response through the priming of naïve T helper cells (Th0) for morphogenesis into effector T cells. In addition, a variety of pro- and anti-inflammatory cytokine molecules produced by DCs in response to pathogen or toxin attack are considered important for differentiation of T cells into autoreactive Th1 lymphocytes, immunosuppressive Th2 cells or regulatory T (Tregs) cells (Banchereau and Steinman 1998). Regulation of these processes is pivotal for maintaining immunological homeostasis in the body (Pulendran, Smith et al. 1999; Pulendran, Kumar et al. 2001; Itano, McSorley et al. 2003; Kapsenberg 2003). In T1D autoimmunity, the maturation of DCs in response to uptake of “self” antigens like the islet β-cell proteins insulin and GAD, induces the development of autoreactive CD8+ cytotoxic T lymphocytes (CTL) and effector CD4+ T helper (Th1) lymphocytes, as well as initiating B cell production of autoantibodies to these islet beta cell proteins. Consequently, in the non-obese diabetic (NOD) mouse, these autoreactive immune cells migrate to the pancreas, infiltrate the islets and secrete inflammatory cytokines such as IFN-gamma and IL-2. These inflammation inducing cytokines are known to stimulate macrophage and CTL secretion of oxidative compounds NO, O₂, H₂O₂ as well as inflammatory cytokines (IL-1 beta, TNF-alpha, TNF-beta) [5-7]. These immune responses are thought to be responsible for induction of chronic pancreatic inflammation (insulitis), which ultimately results in destruction of the islet β-cells and the production of insufficient levels of
insulin to meet the growing body’s metabolic requirements for energy leading progressively to diabetes onset (Piccinni, Beloni et al. 1998).

Recently, our laboratory demonstrated that prevention of T1D onset may be mediated via induction of immunological tolerance resulting from DC interactions with CTB::proinsulin/GAD autoantigen fusion proteins (Odumosu, Payne et al. 2010). Inoculation of human monocyte-derived DCs with CTB-INS and CTB-GAD\textsubscript{35} fusion proteins resulted in suppression of DC maturation. Further, our experimental results showed that CTB-autoantigen fusion proteins modulate DC cytokine secretion profiles in the direction of immunological tolerance while suppressing the biosynthesis of inflammatory cytokines IL-12 that could lead to the onset of autoimmunity (Odumosu, Payne et al. 2010). In response to these observations, the major objective of this study is to analyze the effects of CTB-autoantigen fusion protein stimulated DCs on naïve T cell morphogenesis and proliferation.

**Methods and Materials**

Expression and Purification of CTB-INS and CTB-GAD\textsubscript{35} Fusion Proteins in *E. coli*

DNA fragments encoding 35bp deoxyoligonucleotide located proximal to the carboxyl-terminus of GAD\textsubscript{65} –designated as GAD\textsubscript{35} or a 258bp DNA fragment encoding human proinsulin (INS), were genetically linked to the carboxyl-terminus (309bp) of the cholera toxin B subunit (CTB), to generate the fusion gene CTB-GAD\textsubscript{35} and CTB-INS respectively. The CTB-autoantigen fusion genes were cloned into the (A) configuration of the *E. coli* expression vector pRSET(A) (Invitrogen\textsuperscript{TM}, Carlsbad, CA), under control of the bacteriophage T7 promoter in order to achieve high levels of transgene expression.
The pRSET vector also contained an oligonucleotide encoding 6 histidines immediately upstream of CTB permitting isolation of the transgene product. Selective clones were assessed by DNA sequence analysis to confirm the in-frame linkage of CTB, CTB-INS and CTB-GAD₃₅ DNA fragments (Fig 4.1). Recombinant vectors were then introduced into the E. coli producer strain BL21 (DE3)pLysS (Invitrogen, Carlsbad, CA) by electroporation for nickel affinity column isolation of the recombinant protein (Carter, Yu et al. 2006; Odumosu, Payne et al. 2010).

Recombinant CTB-INS and CTB-GAD₃₅ fusion proteins expressed in E. coli were purified by isolating the 6xHis-tagged protein from lysed cells using metal chelation chromatography. Transformed E. coli strain BL-21, was grown in 250 ml Luria Broth (LB) medium containing ampicillin (100mg/ml) with shaking at 37°C. While still in log phase of growth, protein synthesis was stimulated by addition of 90 mg isopropyl β-D-1thiogalacto-pyranoside (IPTG), (Sigma Chemical Co. St. Louis, MO) to the bacterial culture. After 6 hr continued incubation at 37°C, the bacterial culture was pelleted by centrifugation in a SA-600 rotor for 10 min, at 5,000 rpm and 4°C, in a Sorvall RC5B centrifuge. The cell pellet was resuspended in 1.0 ml / tube of 10 mM HEPES buffer (pH 7.5), containing 100 mM imidazole. The cells were disrupted by sonication (3 x 10 sec bursts), at 10 W, with a Sonic 60 Dismembrator (Fisher Sci. Sunnyvale, CA). The proteins were isolated and purified from the bacterial homogenate using a Maxwell Model 16 robotic protein purification system (Promega Inc.™), according to the protein isolation protocol provided by the manufacturer (Promega Inc., Madison, WI). In order to obtain a pure protein product, the robot employs electromagnetically charged Magne-His Nickel-Iron alloy particles with an affinity for the 6-HIS tag linked to the N terminus of
the recombinant fusion proteins. Imidazole was removed from the protein mixture by
dialysis of the preparation against 2 x 1.0 Liter, 10 mM HEPES buffer (pH 7.5), for 4 hr
at 4°C. The purity of the isolated CTB-INS (~23.4kDa) and CTB-GAD$_{35}$ protein
(~22kDa) were determined by electrophoretic mobility analysis in a 12% polyacrylamide
gel in comparison with protein molecular weight standards. The purified proteins were
confirmed by immunoblot analysis and stored at -20°C until further use.

Isolation and Culture of Monocyte-derived DC from Human Cord Blood

Monocyte-derived dendritic cells (MDDC) were prepared from freshly collected
human umbilical cord blood. The leukocyte fraction of cord blood obtained from normal
healthy placenta donors (following LLU IRB approved protocols), was separated from
red blood cell and platelet fractions by Red blood cell lysis protocol. Briefly, the cord
blood was treated with ACK lysis buffer (0.15 M NH$_4$Cl, 1 mM KHCO$_3$, and 0.1 mM
Na$_2$EDTA) to lyse the erythrocytes. After washing the lymphocytes with Miltenyi buffer
(PBS containing 0.5% BSA and 2mM EDTA), monocytes were isolated from the blood
via MACS (Miltenyi Biotec) using PE-conjugated anti-CD14 and anti-PE microbeads
(BD biosciences, San Jose, CA). The CD14+ monocytes were obtained from the total
lymphocyte fraction by incubation with anti-CD14 PE (Phycoerythrin) (Miltenyi Biotech,
Auburn, CA) for 10 min @ 4°C followed by incubation with anti-PE antibody conjugated
to magnetic microbeads, for 15 min at 4°C. The cells were separated magnetically by
passing them through the MACS column as described by the manufacturer (Miltenyi
Biotech, Auburn, CA) (Devaraj, Glaser et al. 2006; Devaraj, Dasu et al. 2008). The
purity of the monocyte fraction was determined by flow cytometry in a BD FACSCalibur
flow cytometer (BD Biosciences, San Jose, CA.

The isolated monocytes were cultured in 6 cm non-pyrogenic polystyrene culture plates in RPMI 1640 culture medium (Mediatech Inc. Manassas, VA, USA) in a humidified atmosphere of 5% CO₂ at 37 °C (Preprotech, Rocky Hill, NJ). The medium was supplemented with 10% fetal bovine serum (FBS), 1 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 50 ng/ml human recombinant GM-CSF, and 10 ng/ml human recombinant IL-4 (ProSpec-Tany TechnoGene, Rehovot Science Park, Israel). The monocyte cell culture was fed every 2 days by gentle replacement of 50% of the medium with fresh medium. The cell cultures were kept in their original plates until harvested to avoid induction of DC maturation caused by mechanical stress associated with re-plating of the cells. The percentage of monocyte-derived iDCs (M-iDC) was determined directly by flow cytometry based on expression of DC-specific cell surface markers (CD14⁻ HLA-DR⁺ CD11c⁺) after 6 days further incubation.

DC Maturation Assay and Phenotyping

The iDCs were stimulated by the addition of CTB (10ug/ml medium), GAD₃₅ (10ug/ml), CTB-GAD₃₅ fusion protein (20ug/ml) or Phorbol myristate acetate (PMA) and Ionomycin at 10ng/ml medium (PMA+Ionomycin), for 48 hrs at 37 degrees, 5% CO₂. Following the incubation period, the expression of DC surface markers (CD14, CD11c, HLA-DR), indicating their state of activation and maturation was determined by flow cytometry. The gated DC (CD14⁻ HLA-DR⁺ CD11c⁺) cell population was analyzed for the expression of co-stimulatory factors CD86, CD83, CD80, and CD40 DC activation markers (BD Pharmingen, San Jose, CA USA).
Isolation of Naïve T cells and Incubation with Vaccine Inoculated DCs

The leukocyte fraction from approximately 50 ml of human umbilical cord blood obtained from consenting normal healthy placenta donors (following LLU approved IRB protocols) was separated from the red blood cell and platelet fractions by a red blood cell lysis as described above and centrifugation at (2,000 RPM), 4°C, in a Beckman Counter Allegra X-15R centrifuge, equipped with an SX4750 rotor. The CD3+ T cells were positively selected from the cord blood leukocyte fraction using the MACS Pan T Cell Isolation Kit II protocol for indirect magnetic isolation of naïve T cells (Th0) from human blood (Miltenyi Biotec. Auburn, CA). The purified Th0 lymphocytes were found to be > 99% pure, as indicated by flow cytometric analysis. The purified naïve Th0 cells were resuspended at a concentration of 1 X 10^6/ml in RPMI 1640 culture medium (Mediatech Inc. Manassas, VA, USA) supplemented with 10% FBS, 1 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin. Prior to addition to the Th0 cells, the iDCs were stimulated with CTB::INS/GAD fusion proteins as described above for 48 hrs at 37°C.

After the 48 hrs incubation, DCs from each treatment sample were washed twice with PBS and mixed with T cells at a DC:T cell ratio of 1:10, DCs (1X 10^4 cells) + naïve T cells (1 X 10^5 cells). Each of the experimental DC-T cell cultures were incubated in approximately 200ul of RPMI 1640 medium supplemented with 10% of FBS, 1% L-glutamine, and 1% penicillin/streptomycin (Sigma-Aldrich; complete medium)/well in a 96-well microtiter plate for 6 days at 37°C without changing the medium. Early DC mediated T cell activation was assessed after 48 hrs of DC - T cell co-culture, by harvesting the cell mixture from several wells into a 2.0 ml culture tube by centrifugation as already described above and staining the cells with fluorescent labeled T cell markers.
CD3, CD4, CD8, CD25, and CD69. The Treg cell populations were identified by flow cytometry according to the following phenotype: CD4+CD25+IL7R-. The cells were harvested from the DC:Th0 cell co-cultured samples as previously described above and surface stained for T cell markers CD3, CD4, CD8, CD25 and CD127. Intracellular staining for the transcriptional activator Foxp3 was also performed as described below to allow for additional Treg characterization. The proliferation of T cells was measured by dilution of carboxyfluorescein diacetate, succinimidyl ester dye (CFSE) after 6 days incubation at a final concentration of 3µM CFSE and by upregulation of intracellular Ki-67, a transcription factor upregulated in proliferating lymphocytes. (The CFSE dye is incorporated into the cell membrane and becomes diluted upon successive cell divisions).

**Intracellular Cytokine and Ki-67 Measurement**

A total of 10^6/ml T cells were used each intracellular cytokine assay. After 48 hrs DC: T cell co-culture, the cells were incubated for an additional 8 hrs with 3 µM monensin for analysis of intracellular cytokines. Intracellular staining for cytokines was performed according to the manufacturer’s instructions (Caltag, CA). The T cells were washed and surface stained with fluorescently labeled CD4 and CD25 antibodies, fixed, permeabilized, and stained for detection of intracellular cytokines IFNγ, TGF-β, IL-17, IL-2 and IL-10. After washing, the cells were resuspended in 1% paraformaldehyde and analyzed for the presence of intracellular cytokines using the MACSQuant flow cytometer. Intracellular analysis of Ki-67 for cell proliferation was also done as described for intracellular cytokines.
Flow Cytometric Analysis

For examination of DC surface markers, the cells were surface stained with antibodies anti-CD86-FITC, anti-CD83-PE, anti-CD40-FITC, anti-CD80-PE, anti-HLA-DR-PerCP anti-CD14-APC anti-mouse IgG1-FITC, anti-mouse IgG1-PE, anti-mouse IgG1-HLA-DR, and anti-mouse IgG1-APC (BD Pharmingen, San Hose, Ca); 7-Aminoactinomycin D (7-AAD) was used to assess the viability of the collected cells. To analyze T cell populations, anti-CD4-PE, anti-CD127-PE, anti-IL-10-PE, anti-CD3-PerCP, anti-CD8-APC-Cy-7, anti-IFNγ- Bio-SA-APC, anti-IL-4-Biotin-SA-APC, anti-CD25-Biotin-SA-APC, anti-TGFβ-PE-Cy-7 fluorescent tagged antibodies were used. The fluorescent tagged antibodies were obtained from eBioscience Inc, and Biolegend, Inc., San Diego, CA, USA. The fluorescence intensity indicated on all flow histograms is shown on a log scale. An Isotype control was run with each set of samples and was used to define negative and positive cell populations. All samples were analyzed using a Miltenyi MACSQuant flow cytometer (Miltenyi Biotec.).

Statistical Analysis

Statistical analyses of experimental treatment groups were performed using a one-way analysis of variance method (ANOVA). Cell population percentages and fluorescence intensity values were presented as the mean ± 95% confidence interval. A p value of < 0.05 was considered to be statistically significant.
Figure 4.1 Construction of the CTB-INS Fusion Gene and Isolation of the Gene Product. (A). Plasmid map of the E. coli expression vector pRSET A (Invitrogen™, Carlsbad, CA), carrying the CTB-INS or the CTB-GAD<sub>35</sub> fusion gene. (B) Map of the cholera toxin B subunit– proinsulin autoantigen gene fusion CTB-INS (567bp). (C) The cDNA fragment of cholera toxin B subunit (CTB) was fused at the C-terminus to the 5kDa pancreatic islet autoantigen glutamic acid decarboxylase (GAD). The expression vectors pRSET-CTB-INS and CTB-GAD are under the control of the bacteriophage T7 promoter containing an oligonucleotide region encoding 6 histidine amino acid residues immediately 5’ upstream of the CTB DNA sequence. The recombinant plasmids were introduced into the E. coli recipient strain BL21 (DE3) pLysS, for optimum fusion protein expression and for nickel binding mediated isolation of the recombinant protein using a Maxwell 16<sup>™</sup> protein isolation robot (Promega Inc, Madison, WI, USA).
Results

CTB-INS and CTB-GAD$_{35}$ Fusion Protein Inoculated DCs Suppress T cell Activation

We investigated the effect of CTB-autoantigen inoculated DCs on Th0 cell morphogenesis. Allogeneic T cells ($10^6$), preactivated with anti-CD3/CD28 monoclonal antibodies for 48hrs were cultured with CTB, INS, GAD, CTB-INS, CTB-GAD$_{35}$, PMA and Ionomycin inoculated DC for 3 days and analyzed for the expression of CD69 (the earliest inducible cell surface glycoprotein acquired during lymphoid activation). The T cells cultured with CTB-INS and CTB-GAD$_{35}$ inoculated DCs showed a significantly reduced capacity for expression of CD69 activation marker in contrast to Th0 cells cultured with a single autoantigen or PMA+Ionomycin stimulated DCs ($p<0.05$)(Fig 4.2). This experimental data suggests that CTB-autoantigen inoculated DCs appear to suppress early T cell activation.
Figure 4.2: CTB-autoantigen fusion protein inoculated DCs suppress anti-CD3/CD28 Ab stimulated T cell expression of CD69 protein. (A) Representative flow cytometry plot showing the presence of CD4+ and CD8+ T cell populations after gating for CD3+ T cells. (B) FACS plots of T cells expressing CD69+ protein after gating for CD4+ (Top) and CD8+ T cells (Bottom).
Figure 4.2. *Continued.*

(C) Bar graph representing the percent of CD69+ activated CD4+ and CD8+ T cells following incubation of naïve T cells with DCs inoculated with CTB-autoantigen fusion proteins or autoantigen alone. The data presented represent the means and SEM for 4 individual experiments. The asterisk (*) indicates the presence of a statistically significant reduction in T cell synthesis of CD69 T cell activation marker protein (p<0.05) for CTB-autoantigen fusion treated samples in comparison with autoantigen alone treated samples.
CTB-INS and CTB-GAD<sub>35</sub> Fusion Protein Inoculated DCs Induce Generation of T Cells with Reduced Proliferative Capacity

To evaluate the ability of CTB-autoantigen inoculated DCs to inhibit T cell proliferation, CD3+ T cells were co-cultured with CTB, INS, GAD, CTB-INS, CTB-GAD<sub>35</sub> and PMA+Ionomycin following their initial activation with PMA+Ionomycin and anti-CD3/CD28 antibodies for 48 hrs at 37°C. The T cells were labeled with CFSE and KI-67 respectively. As shown in Fig.4.3, Th0 cells co-cultured with CTB-INS and CTB-GAD<sub>35</sub> inoculated DCs consistently inhibited the proliferation of CFSE labeled Th0 cells and the expression of transcription factor KI-67 in comparison with Th0 cells co-cultured with INS or GAD autoantigens alone or with PMA+Ionomycin activated DCs that demonstrated multiple divisions in the presence of CFSE (P<0.05) (Fig 4.3).
Figure 4.3: Shows CD 3+ naïve Th0 cell stimulation following inoculation with CTB-autoantigen inoculated DCs. Naive T cells isolated from umbilical cord blood were incubated with anti-KI-67 antibodies and after 4 days additional incubation in culture medium the cells were analyzed by flow cytometry to assess the degree of cell proliferation based on synthesis of KI-67 protein in CD4+ T helper cells (A), or CD8+ cytotoxic T cells (B). The bars in each graph represent the mean and SEM of three independent experiments for each T cell treatment. Based on the synthesis of KI-67, naïve Th0 cells incubated with CTB-INS/GAD autoantigen fusion protein inoculated DCs showed a significant reduction in proliferative capacity in comparison with PMA and Ionomycin inoculated and anti-CD3/CD28 Ab activated T cells. Asterisks (*) over the bars represent a significant difference in the expression of CD69+ in Th0 cells incubated with DCs inoculated with CTB-autoantigen fusion proteins in comparison with T cells incubated with DCs inoculated individually with INS or GAD autoantigens (p<0.05). (C) To identify the number of cell divisions that occur during T cell proliferation, following incubation with CTB-autoantigen fusion protein inoculated DCs, negative control and CD3+, activated T cell samples were labeled with CFSE dye and after 6 days incubation in culture medium, the individual cultures were analyzed by flow cytometry for incremental reductions in CFSE dye staining intensity signifying the presence of successive cell divisions. The histogram represents reductions in CFSE dye fluorescence intensity commensurate with successive cell divisions detected following proliferation of the T cells in each experimental treatment.
CTB-autoantigens Induce CD4+ T regulatory Cells

In the next set of experiments, we investigated the ability of CTB-autoantigen stimulated DCs to induce suppressor CD4+ regulatory T cells (Tregs), based on the increased recognition of their importance in mediating tolerance and protection from autoimmune diseases (Sakaguchi, Sakaguchi et al. 1995). Therefore, DCs stimulated with fusion proteins or activated with PMA+Ionomycin were cultured with CD3+ T cells previously activated with anti-CD3/CD28 antibodies. After five days incubation with the fusion proteins, the cell cultures were harvested and analyzed for the proliferation of CD4+CD25+IL7- regulatory T cells (Tregs), based on the level of Ki-67 expression (Fig 4.4). Our experimental results showed increase in expansion of Treg populations based on Ki-67 expression in CTB-INS and CTB-GAD_{35} inoculated DCs, was significantly upregulated by 4 fold and 2.5 fold in comparison with INS and GAD_{35} inoculated DC cultured with T cells (p<0.05). However, The expression of Foxp3 was not found to differ among all of the Treg populations gated for the CD4+CD25+IL-7- phenotype.
Figure 4.4: CTB-autoantigen inoculated DCs induce T regulatory cell (Treg) proliferation.

(A) Dot plot showing expansion of Tregs by staining the DC:T lymphocyte mixture with CD4+, CD25+, IL-7R-, and intracellular Ki-67 fluorescent-labeled antibodies. The proliferation of Tregs was measured by flow cytometric determination of the percentage of activated Tregs expressing Ki-67. Differences in Treg population expansion were determined by ANOVA (p<0.05). (B) The bar graph represents the percentage of Treg cells expressing Ki-67 transcription factor as an indicator of cell division. The columns represent the means and SEM of three independent experiments. The asterisks (*) represent significant differences in the expression of Ki-67 (cell proliferation) in Tregs cells for T cells incubated with CTB-autoantigen fusion protein inoculated DCs in comparison with T cells incubated with DCs inoculated with the single autoantigens alone (p<0.05).
CTB-autoantigen Stimulated DCs Direct Cognate T cells to Increase IL-10 or TGF-β Cytokine Production

To examine the ability of CTB-autoantigen stimulated DCs to modulate T cell cytokine production, CD3+ T cells were co-cultured with CTB, INS, GAD, CTB-INS, CTB-GAD35 or PMA+Ionomycin inoculated DC, following initial activation of the naïve T cells with anti CD3/CD28 antibody and PMA+Ionomycin. Intracellular cytokine production in the DC inoculated T cells was determined following incubation of the cultures in medium containing 3 µM monensin for the last 8 hrs of the incubation period. In comparison with INS and GAD35 inoculated DC treated T cells, both CTB-INS and CTB-GAD35 stimulated T cells produced significantly higher amounts of immunosuppressive IL-10 and TGF-β cytokines (p<0.05) and significantly reduced amounts of proinflammatory IFN-γ, IL-2 and IL-17 (p<0.05). Incubation of naïve T cells with DCs activated by CTB-INS induced significantly higher levels of IL-10 biosynthesis, while T cells incubated with DCs activated by CTB-GAD35 fusion protein resulted in significantly increased levels of TGF-β biosynthesis (P<0.05). Taken together, these experimental results suggest that CTB-autoantigen inoculated DCs may induce differentiation of immunosuppressive Th2 and Treg populations with CTB-INS and CTB-GAD35 inoculated DCs stimulating T cell biosynthesis of cytokines normally synthesized by Tr1 and Th3 Tregs respectively. These experimental results indicate that CTB-autoantigen inoculated DCs phenotype are likely associated with immunological tolerance and T cell regulatory activities.
Figure 4.5: CTB-autoantigen stimulated DCs direct cognate T cells to increase IL-10 and TGF-β anti-inflammatory cytokine production. The CD3+ T cells isolated from umbilical cord blood were co-cultured with CTB, INS, GAD, CTB-INS, CTB-GAD_{35} or PMA+Ionomycin, following initial stimulation of the naïve T cells with PMA+Ionomycin +antiCD3/CD28 antibodies for 48 hrs. Monensin (0.3 μM), was added to each cell culture sample during the last 8 hrs of incubation to permit accumulation of cytokine levels within the DC incubated T cells prior to flow cytometric measurements of sequestered T cell cytokine levels. (A) The overlapping histograms show T cell populations synthesizing IL-10, TGF-β, IFN-γ, IL-2 and IL-17 cytokines following T cell incubation with DCs previously inoculated with CTB, INS, GAD, CTB-INS, CTB-GAD_{35} or PMA+Ionomycin in comparison with the control cultures (T cells incubated with immature DCs). The CTB-INS and CTB-GAD_{35} stimulated T cell samples synthesized significantly higher amounts of immunosuppressive IL-10 and TGF-β cytokines (p<0.05) and significantly diminished amounts of proinflammatory IFN-γ, IL-2 and IL-17 (p<0.05). (B) The graphs indicate mean fluorescence intensity levels representing T cell biosynthesis of IL-10, TGF-β, IFN-γ, IL-2 and IL-17 cytokines following their incubation with DCs inoculated with the autoantigen fusion proteins described above. Statistical significance among the T cell cultures was calculated based on P<0.05.


**Discussion**

Previous studies have demonstrated the major role played by DCs in the induction and maintenance of immune tolerance (Steinman, Turley et al. 2000; Wakkach, Fournier et al. 2003). The results of our previous CTB-INS animal studies have established a molecular basis for CTB-INS and CTB-GAD\textsubscript{35} fusion proteins in suppression of Type 1 diabetes (T1D) onset in NOD mice (Denes, Krausova et al. 2005; Gong, Jin et al. 2007; Gong, Long et al. 2009). In these studies, we demonstrated that the fusion of CTB protein to the autoantigen was required to achieve a dramatic enhancement of immune suppression. Following these animal studies, our human ex vivo studies revealed that co-delivery of CTB + the autoantigen did not result in a significant inhibition of DC activation and maturation (Odumosu, Payne et al. 2010). Taken together our experimental findings suggest that linkage of CTB to the autoantigen is key to the identification of the mechanism underlying adjuvant-enhanced autoantigen mediated immunological tolerance. However, cellular and molecular mechanisms underlying multi-component vaccine suppression of T1D remain unidentified. The dominant role of DCs in the decision required to induce inflammation or to activate immunotolerance implicates this APC resides at the point of earliest initiation of CTB-autoantigen mediated suppression of diabetes onset. To confirm this observation, we assessed the interaction and outcome of CTB-autoantigen stimulated DCs on naïve T cells. Our experimental data suggests that CTB-autoantigen inoculated DCs induce the ex vivo differentiation of IL-10 and TGF-β producing T cells that possess limited proliferative capacity. This experimental result is in agreement with previous experiments describing the immunosuppressive nature of Th2 and regulatory Tr1 and Th3 cells (Levings, Gregori
et al. 2005; Battaglia, Stabilini et al. 2006; Roncarolo, Gregori et al. 2006).

In this ex vivo study, human monocyte-derived DCs incubated with CTB-autoantigen fusion proteins generate Tregs from CD3+ T cells. In comparison with Tregs incubated directly with INS or GAD35 autoantigens alone, T cells incubated with CTB-INS/GAD inoculated DCs generated more than a 2.5 fold increase in proliferation compared to control samples and PMA+Ionomycin inoculated samples. These experimentally identified immunosuppressive properties are in agreement with those described for the performance and functions of Tr1 and Th3 regulatory T cells.

Traditionally, a marker for specific identification of Tregs is expression of the forkhead box transcription factor (FOX) P3, possibly because foxp3 gene transfer was shown to convert naïve CD4+CD25- T cells into functional regulatory T cell populations (Fontenot, Gavin et al. 2003; Hori, Nomura et al. 2003; Khattri, Cox et al. 2003). While FOXP3 protein biosynthesis can be used to quantify Treg populations and assess their purity, FOXP3 is an intracellular protein that requires cell fixation and permeabilization prior to detection. This requirement makes it impossible to use FOXP3 as a marker for identification of live Treg cell populations for functional studies. Of particular interest would be the identification of a cell surface marker that correlates with FOXP3 and represents a specific marker for regulatory T cells that is independent of Treg CD25 expression. Earlier reports by Liu et al. in 2006 (Liu, Putnam et al. 2006) and Seddiki et al (2006) showed that differential expression of CD127 (the α-chain of the IL-7 receptor) enables separation of human CD4+CD25+CD127- Tregs based on flow cytometry. Both studies indicated that CD127- cells were FOXP3 positive in human peripheral blood and that CD127 was a better marker than CD25 in mice, with most CD4+Foxp3 Treg cells
having a CD127- phenotype. In addition, the functional immunosuppressive activity of CD127- Treg populations and its absence in the presence of the CD127+ marker protein was also clearly demonstrated (Liu, Putnam et al. 2006). In this study, we have identified Treg cell populations based on flow cytometric identification of the CD4+CD25+CD127- phenotype, the cytokines that they synthesize and their proliferative capacity (a sign of their expansion in response to DC activation). In confirmation of the observations of Seddiki and Liu, the CD4+CD25+CD127- Treg populations analyzed in our study were also found to be Foxp3+ (data not shown).

Several observations suggest that Tr1 cells are involved in CTB-INS/GAD maintenance of DC –T cell mediated peripheral tolerance *ex vivo*. Certain regulatory T cell subsets (Tr1) play an important role in modulating immune responses during the progression of autoimmunity, based on the reduction in IL-10 producing CD4+ cells observed in the synovial tissue and peripheral blood of rheumatoid arthritis and autoimmune hemolytic anemia patients (Yudoh, Matsuno et al. 2000; Hall, Ward et al. 2002).

Perhaps the most significant class of novel effector cell types involved in both human and mouse organ specific autoimmunity models are the recently identified Th17 cells that secrete the pro-inflammatory cytokine IL-17 (Fujino, Andoh et al. 2003; Nakae, Nambu et al. 2003; Komiyama, Nakae et al. 2006). This inflammatory cytokine was first detected in the serum and tissues of patients with autoimmune diseases such as multiple sclerosis (MS), systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) (Wong, Ho et al. 2000; Tzartos, Friese et al. 2008). Blockade of IL-17 was shown to prevent the development of Experimental Autoimmune Encephalitis (EAE), while IL-17 knockout
mice showed resistance to the development of EAE and collagen-induced arthritis (CIA) (Nakae, Nambu et al. 2003; Hofstetter, Ibrahim et al. 2005). These experimental results suggest that regulation of Th17 differentiation and function may prove to be a potential therapeutic target for organ specific autoimmune diseases. Our recent experiments identified CTB-GAD\textsubscript{35} suppression of DC synthesis and secretion of IL-6, a pleiotropic cytokine involved in regulating the balance of IL-17 producing Th17 cells and Treg cells (Bettelli, Carrier et al. 2006; Mangan, Harrington et al. 2006). Further, IL-6 was found to be an effective target for down-regulating the differentiation of Th17 cells as a therapeutic treatment for autoimmunity (Nishimoto, Terao et al. 2008; Yokota, Imagawa et al. 2008). In this study, our experimental findings demonstrate that CTB-INS and CTB-GAD\textsubscript{35} suppress T cell biosynthesis of IL-17. This experimental result in combination with CTB-GAD\textsubscript{35} inhibition of DC IL-6 biosynthesis may be critical elements in the elucidation of cellular mechanisms responsible for CTB-autoantigen mediated immune suppression of T1D autoimmunity.

In conclusion, this study supports the hypothesis that CTB-autoantigen fusion protein modulated DCs stimulate the morphogenesis of immunosuppressive human T cells \textit{ex vivo}, largely through inhibition of DC maturation, synthesis of inflammatory cytokines and by stimulation of DC biosynthesis of anti-inflammatory cytokines. Although many experimental strategies have been adopted to induce or expand immunosuppressive cells to inhibit the immune responses underlying autoimmune pathologies, immunological tolerance can be maintained by selected adjuvant autoantigen fusion vaccine administration through the induction of the anti-inflammatory cytokine IL-10 (Arakawa, Chong et al. 1998). In a separate \textit{in vivo} study, our laboratory recently
demonstrated that a combinatorial therapy of CTB-GAD$_{35}$ + IL-10 is able to completely suppress the onset of type 1 diabetes in NOD mice (Denes, Fodor et al. 2010). Our experimental results suggest that ex vivo inoculation of human DCs with adjuvant – diabetes autoantigen fusion proteins favors immunological suppression of diabetes onset and suggests that mechanisms of immunological tolerance responsible for multi-component vaccine suppression of diabetes onset are comparable in humans and animals and most importantly provides a readily available ex vivo experimental system closely resembling in vivo conditions for analysis of improved therapeutic strategies for resolution of human diabetes autoimmunity. Experiments involving CTB-INS/GAD vaccine suppression of previously activated DCs may provide ex vivo evidence that the progression of human T1D can be safely halted to re-establish immunological homeostasis and thereby set the stage for pancreatic stem cell repopulation of the islets of Langerhans with insulin secreting “beta-like” cells that may persistently alleviate T1D in human patients.

**Acknowledgments**

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References


CHAPTER 5

CONCLUSIONS

The establishment of individual autoantigen mediated immunotolerization strategies has provided a basis for assembly of individual therapeutic molecular components into a multi-component vaccine for immunological suppression of Type 1 Diabetes (T1D). These multi-component vaccine strategies appear at first glance to be safer, more effective in immune suppression and more durable than application of the individual vaccine components alone. Early experimentation with individual molecular therapeutic strategies was shown to provide short-term suppression of organ specific autoimmunity (Weiner, Zhang et al. 1991; Zhang, Davidson et al. 1991). However, few of these individual therapies were shown to demonstrate effective, safe and persistent protection against disease onset and progression (Hutchings and Cooke 1998). When linked to a pathogen or foreign antigen, the weak protein adjuvant cholera toxin B subunit (CTB) was often found to stimulate antigen-specific mucosal immunity and to strengthen immune responses against pathogen proteins. Experimental evidence suggested that CTB stimulation of innate immunity was initiated through activation of immature dendritic cell (iDC) signal transduction pathways that lead to DC maturation. The stimulation of DC maturation by CTB resulted in DC synthesis and secretion of inflammatory cytokines and chemokines such as IFN-γ, IL-2 and CTLA4 responsible for DC migration to the spleen or peripheral lymph nodes where DC secreted inflammatory cytokines that assisted in the guidance of cognate naïve Th0 helper cell differentiation
into immunostimulatory effector Th1 lymphocytes and cytotoxic T cells.

However, when CTB was linked to an autoantigen such as insulin in T1D, it often stimulated autoantigen mediated suppression of organ specific autoimmunity many fold, achieving levels of immunological tolerance approaching that required for clinical intervention. This exception of CTB–autoantigen fusion protein enhancement of immune suppression to the expected paradigm of enhanced immunity emphasizes the novelty of the CTB as an immunomodulatory molecule for development of therapeutic vaccines. These apparent counter-intuitive attributes emphasize the extent to which the mechanisms underlying CTB stimulation and suppression of immunity remain as yet under studied. Combinatorial vaccines involving oral delivery of the CTB linked to pancreatic autoantigens such as proinsulin and GAD, were shown to provide significant increases in enhancement of autoantigen mediated suppression of Type 1 diabetes symptoms in contrast with inoculation of prediabetic mice with glutamic acid decarboxylase (GAD) or proinsulin alone (Arakawa, Chong et al. 1998). More recent combinatorial DNA vaccination experiments including genes encoding CTB-GAD in combination with the immunosuppressive cytokine IL-10 have demonstrated complete suppression of diabetes onset in non-obese prediabetic (NOD) mouse model of T1D. Thus, it is now clear that combinatorial vaccination strategies can completely prevent diabetes onset in genetically susceptible mammals with no detectable undesirable effects. The experimental studies presented here emphasize the possibilities for increased vaccine effectiveness in suppression of diabetes onset based on the development of combinatorial vaccination strategies.

In chapters 2 and 3 of this dissertation, the experimental data suggest that fusion
of CTB to autoantigens appears to activate genes that stimulate DC synthesis of anti-inflammatory cytokines such as IL-10 that suppresses DC maturation in combination with inhibition of DC biosynthesis of co-stimulatory factors CD86, CD83 and CD80 required for naïve Th lymphocyte activation. Further, CTB-autoantigen fusion protein inoculated DCs were shown to secrete reduced levels of inflammatory cytokines IL-12 and IL-6 while at the same time upregulating biosynthesis of the anti-inflammatory cytokine IL-10. In chapter 4, the incubation of DCs with adjuvant-autoantigen fusion proteins was found to enhance their guidance of naïve Th0 cell morphogenesis and proliferation into anti-inflammatory Th2 lymphocytes and regulatory T cells capable of inhibiting diabetes onset. In addition, it became clear from the cytokine analysis studies that multi-component vaccine suppression of DC maturation lead to the suppression of autoreactive Th1 and Th17 inflammatory lymphocyte development, suppressing their potential for initiation of T1D insulitis and hyperglycemia. These ex vivo human immune cell vaccination experiments establish a basis for identification of mechanisms responsible for vaccine inhibition of human diabetes progression and the way for in vivo analysis of multicomponent vaccine strategies for rescuing the large numbers of diabetes patients in which the disease has already progressed to hyperglycemia.

Further, these experiments establish a basis for development of safer and more effective treatments for supplementation or replacement of current nonspecific immunosuppressants, anti-inflammatory steroids or interventional therapies like islet transplantation, which can increase patient vulnerability to infection and other complications. These experiments provide a basis for development of safer and more effective vaccine strategies for preventing diabetes onset and progression in patients that
can lead to restoration of immunological homeostasis and euglycemia in T1D patients.

Several studies have reported that oral administration of autoantigens may actually exacerbate autoimmune disease (Genain, Abel et al. 1996; Bellmann, Kolb et al. 1998; Blanas and Heath 1999). Although no instances of autoimmune disease have been reported following application of recombinant protein, experiments remain to be performed that will determine the optimum dose and frequency of immuno-modulated autoantigen delivery required to achieve the maximum protective efficacy.

An immediate major benefit of CTB adjuvant enhanced vaccine technology presented in this dissertation will be delivery of mucosal subunit vaccines for protection of populations in countries suffering health care disparities. To this end, our group has focused on a promising approach to the production of an autoimmune disease protective vaccine generated in edible transgenic plants (Carter, Odumosu et al.; Chong, Roberts et al. 1997; Arakawa, Yu et al. 1998; Arakawa, Yu et al. 1999). One of the major limitations of this is the possibility for initiation of autoimmune disease symptoms in disease-free individuals who accidentally consume autoantigens linked to a strong bacterial adjuvant. Ethical issues regarding recombinant DNA mediated immunotherapy are more likely to subside as the public becomes more adequately informed concerning the safety of genetically modified organisms (GMO). With each successful clinical trial, confidence in the expediency of using edible vaccines for disease prevention in the population will increase in the scientific, corporate and private sectors.

Nonetheless, the molecular and biochemical studies presented within this doctoral dissertation, demonstrate that CTB-INS/GAD suppression of autoimmune responses provides a basis for development of pluripotent adult or totipotent embryonic stem cell
repopulation of the pancreas with insulin producing beta cells for restoration of
euglycemia and return of the T1D patient to a durable state of immunological
homeostasis. The further establishment of complete immune suppression of diabetes will
help to ensure both preventive and interventional therapy for T1D in patients suffering
from this life-long chronic inflammatory autoimmune disease. This application in
combinatorial vaccination protocols may prove to be a plausible direction for effective
immune suppression of additional organ-specific autoimmune diseases such as, Graves's
disease, Hashimoto’s thyroiditis, rheumatoid arthritis, multiple sclerosis, myasthenia
gravis and uveitis.
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APPENDIX A

ISOLATION OF LEUKOCYTES FROM CORD BLOOD

Collection of umbilical cord blood from Cesarean section patient at Loma Linda University Medical Center:

Cord blood is collected from patients who had just undergone Caesarean section surgical procedure. 25 ml of cord blood is usually collected by Inevely Baez in a 50ml conical tube under specified IRB approved protocols. Stem cells CD34+ cells which make up 1% of PBMNC are isolated by Magnetic Separation using Micro beads. The remaining cell populations which are CD34- are frozen in Growth Media containing RPMI 1640 supplemented with 10% FBS, 1 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin (D10).

Procedure
I. Note time blood collected in notebook.
II. Dilute CB 50/50 with Hanks (HBSS) in 50 ml tube.
III. For each full tube of Hanks/CB mixture prepares two 50 ml tubes containing 25 ml of Ficol.
IV. Gently layer 25 ml of Hanks/CB mixture over Ficol being careful not to disturb the interface between the blood and the Ficol (holding tube at an angle may help).
V. Spin in centrifuge at room temp, 2000 RPM; brake off, for 30 minutes.
VI. Remove tubes from centrifuge being careful not to disturb buffy layer of mononuclear cells that are located at the interface between the serum (on top) and the Ficol (on bottom)
VII. Using a pipette remove and discard serum and from top of buffy layer leaving about 1/4 to 1/2 inch of serum on top of off buffy layer.
VIII. Place pipetter at the top of serum and remove remaining serum along with all of buffy layer and transfer to a clean 50 ml tube. Be sure that pipetter is at surface of liquid Ficol is not aspirated from under buffy layer. Add Hanks to fill tube to top.

IX. Spin in centrifuge at room temp, 1500 RPM, brake on low, for 10 minutes.

X. Aspirate off supernatant. Resuspend cells in 10 mls of PBS and then transfer cells to pellet in next tube. Resuspend cells and transfer to next tube, until all cells are in one 50 ml tube. Wash remaining cells from empty tubes by repeating the process with 10 additional mls and which should be placed in 50 ml tube with cells.

XI. Fill 50ml tube with cells to top with PBS and spin in centrifuge at room temp, 1500 RPM, brake on low, for 10 minutes.

**IF FICOLL LAYER WAS EXTREMELY BLOODY PERFORM ORTHOLYSIS (SEE BELOW) AT THIS POINT.**

XII. Aspirate off supernatant and resuspend cells in 10 ml of PBS. Transfer to 15 ml tube. Wash 50 ml tube with 1 ml of PBS and transfer wash to tube with the rest of cells.

XIII. Count by diluting 10 ul of cells in 990 (1:100) or 90 (1:10) ul of 3% acetic acid.

XIV. Spin remainder of tube in centrifuge at room temp, 1500 RPM, brake on low, for 5 minutes.

XV. Aspirate supernatant off cells, and resuspend in 300 ul of Miltenyi buffer per 10^8 cells.

XVI. Add 100 ul of solution A1 per 10^8 cells counted in step 13 above and incubate at 4°C for 15 minutes.

XVII. Add 100 ul of Solution A2 per 10^8 cells and incubate an additional 15 minutes.

XVIII. Add Miltenyi buffer to 10 ml and spin down (5 minutes at 1500 RPM), aspirate off buffer.
XIX. Resuspend in 400 ul Miltenyi buffer per $10^8$ cells and add 100 ul of reagent B (beads) per $10^8$ cells and incubate at 4 $^\circ$C for 15 minutes.

XX. Add Miltenyi buffer to 10 ml, spin down and pull off supernatant. Resuspend cells in 1 ml of Miltenyi buffer very well using 1 ml pipet.

XXI. While beads are spinning set up magnetic apparatus: --Place column in magnetic (2 prongs out) being careful to keep tip of column and plunger sterile.--place pre-filter on top of column--Add .5 ml of Miltenyi buffer to prefilter and pipette up and down until liquid goes through to column . Let drip through column to 15 ml centrifuge tube set up in Styrofoam rack.

XXII. Add cell suspension to top of column and let cells run through.

**COLLECT RUN THROUGH (CD34-CELLS) IN A CLEAN TUBE**

XXIII. **TO ELUTE CD34+ CELLS:** Wash 3 times by adding .5 ml of buffer to top of column and letting run through.

XXIV. Get 1.2 ml of buffer in pipetman and place in column while holding it over 4 ml collection tube. Being careful to keep end of plunger sterile put in top of column and plunge down.

XXV. If high purity needed plunge cells into a second column which has been placed in magnet.

Ortholysis

Aspirate off supernatant. Lyse red blood cells by resuspending cells in 10 ml of lysis buffer and incubating for 10 minute at 37 $^\circ$C (water bath). After incubation fill tube to top with HBSS.

Spin down cells (5 minutes at 1500 RPM), aspirate off supernatant, resuspend cells in 10 ml of PBS and transfer to 15 ml tube. Dilute a small sample 1:10 in PBS for counting. Continue at step 14 in above protocol.
TIME: 5-6 hours at first, later 3-4 hours

NOTE: From one tube of cord blood can get
~1 X 10^8 white blood cells
1-2 X 10^6 CD34+ (approximately 1% of cord blood)
1 X 10^4 CD38- CD34+

MATERIALS:

Red Blood Cell Lysis Buffer

Dissolve the following in 800ml distilled H_2O

- 8.3g NH_4Cl
- 1.0g KHCO_3
- 1.8ml of 5% EDTA

Filter sterilize through 0.2um filter

Bring volume to 1000ml with distilled H_2O

Miltenyi buffer (Mini MACS buffer) --.5% BSA, 5 mM EDTA, PBS
For 250 ml: 12.5 ml 10% BSA--culture grade (or 16.7 ml of 7.5% BSA)
2.5 ml of 0.5 M EDTA
1X PBS to 250 ml

Filter-sterilize and then wrap parafilm around top of filter to seal degas for 1 hour prior to use. Store tube at 4°C.
APPENDIX B

ISOLATION OF CD14+ MONOCYTES FROM CD34- CORD BLOOD

Frozen cord blood CD34-cells are thawed in thawing medium containing 25 ml of any medium containing at least 10% serum (D10, R10, etc), 100 units of sterile heparin (100 ul of 1000 U/ml stock) and 750 Units of DNAase (250 ul of 3000U/ml stock Sigma #D-5025 in water) For 150,000 Unit bottle dissolve in 50 ml of sterile water and aliquot.

Procedure:

I. Thaw one 2 ml vial of frozen cells quickly in water bath.
II. Rinse vial with alcohol and dry.
III. Place cell suspension in 50 ml centrifuge tube.
IV. Add 25 ml of thawing medium drop by drop swirling after each addition. Should take about 2-3 minutes to dilute sample.
V. Place cells at 37°C for 1 hour.
VI. Spin down cells in centrifuge and then wash with 5 ml PBS or Miltenyi buffer (Bechmann Counter Allegra X-15R centrifuge, SX4750 RPM 1500 for 8 min @ 4°C).
VII. Aliquot 10 ul of cells for counting.
VIII. Count cells by hemocytometer (diluting 1:2 with trypan blue) multiply by appropriate volume: (# cells in one frame) X (trypan blue dilution—usually 2) X (10^4) X (# of mls of cells)
IX. Aspirate off supernatant after washing cells. Transfer cells to a FACs tube and wash again in 2 ml Miltenyi Buffer (5 minutes @ 1500 RPM).

X. Aspirate off supernatant.

XI. Resuspend cells in 100 ul of Miltenyi Buffer for each 50 million cells.

XII. Add 10 ul of CD14-PE antibody for each 100 ul of cell suspension.

XIII. Incubate 10 minutes in Fridge.

XIV. Add 4 ml Miltenyi Buffer to fill the tube.

XV. Aspirate supernatant and resuspend at 5X10^7 cells per 80 ul of Miltenyi buffer. Add 20 ul of anti-PE microbeads (make sure beads are mixed well—do not vortex) per 80 ul of cells. Incubate for 15 minutes. Fill tube with Miltenyi buffer and wash.

XVI. Aspirate off supernatant and resuspend cells (2 mls) for separation by MACs column or other means. (SEE BELOW)

XVII. Take a small aliquot of separated cells for FACs.

XVIII. Wash remaining cells. Aspirate off supernatant and resuspend in medium for generating DCs as per paper.

MACS MAGNETIC SEPARATION

I. Place column in the magnetic field of a suitable MACS Separator. For details see the respective MACS Column data sheet.

II. Prepare column by rinsing with the 3 ml Miltenyi buffer

III. Apply cell suspension onto the column.

IV. Collect unlabeled cells that pass through and wash column with the 3X 3 ml Miltenyi buffer. Collect total effluent; this is the unlabeled cell fraction. Perform washing steps by adding buffer three times. Only add new buffer when the column reservoir is empty.
v. Remove column from the separator and place it on a 15 ml conical collection tube.

vi. Pipette 5ml Miltenyi buffer onto the column. Immediately flush out the magnetically labeled cells by firmly pushing the plunger into the column. *(CD14+ MONOCYTES)*

VII. (Optional) To increase the purity of the magnetically labeled cells, the eluted fraction can be enriched over a second MS or LS Column. Repeat the magnetic separation procedure as described in steps 1 to 6 by using a new column.

VIII. Aliquot 10 ul of cells for counting (this is to determine how many cells will be plated per well for DC cell culture.

IX. Aliquot 100ul for FACS determination of purity (compare to sample before magnetic separation.
APPENDIX C

DIFFERENTIATION OF DCs FROM MONOCYTES

Adapted from Agrawal et al. *JI* 2007 178: 6

Medium

- R10 medium
- 50 ng/ml rhGM-CSF
- 10 ng/ml IL-4

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**R10 MEDIUM**

- FBS (tested to be good for B cell cultures) 550 ml
- Penn/Strep 550 ml
- L-glutamine 550 ml
- 2-ME (10^{-1} M stock) must have for B cells
- RPMI 550 ml

---

~560 ml

Use of GM-CSF

- Prospec GMCSF = $50 for 2 ug
- 2ug is enough for 200 ml of medium
- Stock: Resuspend 2 ug in 1 ml = 2 ng/ul
- So add 25 ul of stock per ml of medium

Use of IL-4

- Prospec IL-4 = $50 for 2 ug
- 2ug is enough for 200 ml of medium
Stock: Resuspend 2 ug in 1 ml = 2 ng/ul
So add 5 ul of stock per ml of medium

**Procedure:**

I. In 4 ml of Growth Media, add 100 ul of stock GM-CSF and 20ul IL-4 of medium

II. Spin down cells in Miltenyi buffer after counting and completely aspirate supernatant.

III. Plate cells at 1 X 10^5 cells per ml of culture medium in 24 well plate.

IV. Remove and replace half of medium every 2 days for 6 days.
APPENDIX D

EXPERIMENTAL SET-UP FOR DENDRITIC CELL MATURATION AND T-CELL FUNCTIONAL STUDIES

DC+ Fusion protein → 48hrs → DC+ Fusion protein → 48hrs → DC+ Fusion protein

Add Monensin
Add PMA+Ionomycin

6-8hrs

Measure Intracellular cytokine synthesis by flow cytometry
IL-4, IL-10, TGF-B, IFN-Gamma, IL-6 and IL-17A
### STIMULATION OF DCS WITH FUSION PROTEINS

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Remark</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. No Stimulation</td>
<td>Negative control</td>
</tr>
<tr>
<td>2. CTB</td>
<td>Use @10ug/ml (initial conc. is 166.6 ug/ml) Therefore use @ 60.02 ul per ml of growth media</td>
</tr>
<tr>
<td>3. INS</td>
<td>Use @5ug/ml (stock soln is 0.5mg/ml) Therefore use @ 10ul/ml</td>
</tr>
<tr>
<td>4. GAD</td>
<td>Use @5ug/ml (initial conc. is 166.6 ug/ml) Therefore use @ 30.01 ul per ml of growth media</td>
</tr>
<tr>
<td>5. CTB-INS</td>
<td>Use @20ug/ml (initial conc. is 2.5ug/ml) Therefore use @ 4 ul per ml of growth media</td>
</tr>
<tr>
<td>6. CTB-GAD</td>
<td>Use @10ug/ml (initial conc. is 166.6 ug/ml. Therefore use @ 60.02 ul per ml of growth media</td>
</tr>
<tr>
<td>7. CTB + INS</td>
<td>Use CTB @10ug/ml (initial conc. is 83.3 ug/ml) Therefore use @ 30.01 ul per ml of growth media Use @10ug/ml (stock soln is 0.5mg/ml). Therefore use at 5ul/ml</td>
</tr>
<tr>
<td>8. CTB + GAD</td>
<td>Use CTB @10ug/ml (initial conc. is 166.6 ug/ml. Therefore use @ 30.01 ul per ml of growth media Use GAD @10ug/ml (initial conc. is 166.6 ug/ml. Therefore use @ 30.01 ul per ml of growth media</td>
</tr>
<tr>
<td>9. CTB-INS + CTB-GAD</td>
<td>Use CTB-INS @10ug/ml (initial conc. is 2.5ug/ml). Therefore use @ 2 ul per ml of growth media Use CTB-GAD @10ug/ml (initial conc. is 166.6 ug/ml. Therefore use @ 30.01 ul per ml of growth media</td>
</tr>
<tr>
<td>7. PMA + Ionomycin</td>
<td><strong>Positive Control</strong> Use PMA at 10ng/mL of the cell suspension. Ionomycin use at conc. 1mg/mL.</td>
</tr>
</tbody>
</table>

---

**a. Wash cells with PBS and spin down for 5 minutes**
**DC-T cell Co-culture setup**

a. Isolate T lymphocytes from cord blood using anti CD3-PE beads  
b. Co-Culture DC-T cell (1:10 ratio) for 48 hrs

<table>
<thead>
<tr>
<th>Add anti Cd3/Cd28 activation beads to T cells except last sample</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>No stim DC only</strong></td>
</tr>
<tr>
<td>CTB-DC + T cell</td>
</tr>
<tr>
<td>LPS-DC + T cells</td>
</tr>
<tr>
<td>CTB + INS-DC + T cells</td>
</tr>
</tbody>
</table>

After 48 hrs, remove media and wash cells with PBS

**Perform Golgi accumulation of Cytokine Assay**

1. Monensin on ice
2. Remove plate from incubator after 48 hrs
3. Add (1ul/ 200ul of media) of 3 mM monensin to each well
4. Return to incubator for 6hr
5. Final Concentrations are 15mM monensin (In addition to monensin, ADD PMA and Ionomycin to cells)

**After 6 hrs**

Harvest cells from each well and individually labeled FACs tube.

Wash in 2 ml PBS

Spin down cells pour off supernatant and make sure that cells are in 10ul staining
wash.

Transfer to 96-well plates for staining

**Surface Stain:** CD4PB, CD127PE, CD8 E-flour antibodies: 2 ul per; APC antibodies .5 ul per well.

**Incubate 15 minutes**

Wash with Staining Buffer and resuspend in 10ul staining buffer

STAIN WITH 1:50 DILUTION OF SECONDARY SA-PE CY7

• STAIN ISOTYPE CONTROL AT THIS TIME

Incubate 15 minutes

**Intracellular cytokine staining following Surface staining using CALTAG METHOD**

**Note:** **DO NOT RESUSPEND IN 1% PARAFORMALDEHYDE AFTER SURFACE STAINING**


2. **Incubate** for 15 minutes at room temperature

3. Wash cells with staining buffer (200ul, spin and flick)

4. Add 20ul of reagent B (permeabilization solution) to each well.

5. Add antibodies to each well and pipette up and down

6. **Incubate** for 20 minutes at room temperature.

7. Wash cells with staining buffer

8. Resuspend in 200ul 1% paraformaldehyde
APPENDIX E

LINEAGE MAP OF LUEKOCYTE DIFFERENTIATION

Hematopoietic Stem Cells

Myeloid Precursor

- Monocytes
- Neutrophils
- Basophils
- Eosinophils

Macrophages

Dendritic Cells

Lymphoid Precursor

- B Cells
- T Cells
- Natural Killer Cells