Regulatory T cell plasticity and its role in the rejection of pancreatic islet allograft tissue

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http://hdl.handle.net/2144/16174

Boston University
REGULATORY T CELL PLASTICITY AND ITS ROLE IN THE REJECTION OF PANCREATIC ISLET ALLOGRAFT TISSUE

by

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B.S., College of Charleston, 2013

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

2015
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ACKNOWLEDGMENTS

First and foremost, I would like to thank the many people whose help and guidance have been essential in bringing me to this point. To my mentor and Principal Investigator, Dr. Maria Koulmanda, as well as Dr. Terry Strom: thank you for giving me the wonderful opportunity to work in your lab. I could never have imagined ending up in such a wonderful place to work. This has been a hugely educational experience and has left me with a great passion for research. You placed a lot of trust in me and I cannot possibly thank you enough.

To Dr. Thomas Thornley: thank you for your endless guidance. Truly, these experiments could not have happened without your tremendous help. Your kindness and willingness to help made this process thoroughly enjoyable. Thank you for your patience, mentorship, and great ability to teach.

To my advisor, Dr. Linda Heffner, thank you for all you have done for me these past two years. I could not have ended up with a better advisor. Your guidance and expertise is second to none. You have been a tremendous help in this journey, and I cannot thank you enough.

I would like to thank Dr. Vaja Chipashvili and Dr. Zhigang Fan for their invaluable assistance with islet isolations and transplantations. I would also like to express my gratitude to Lingzhi Ma for her expertise with PCR. Thank you Vasilis Toxavidis, John Tigges, and Ginny Camacho for your help with flow cytometry and cell
sorting. I would also like to thank Dr. Periklis Kyriazis, Dr. Krishna Agarwal, and Dr. Sarantis Korniotis for their general support in this study.

Thank you everyone for always being there to help with this project. What a great journey this has been!
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ABSTRACT

The healthy immune system is a delicate and precisely orchestrated balance between activation and suppression. It is well established that regulatory T cells (Tregs) have substantial immunosuppressive properties and play a pivotal role in maintaining this balance. Many autoimmune states have been characterized by disproportionately high numbers of T effector cells, and comparatively low numbers of regulatory T cells (Hori et al., 2003; Sakaguchi et al., 1995; Choileain et al., 2006). Furthermore, mouse models in which regulatory T cells are removed or rendered ineffective show rapid development of autoimmunity. It is therefore hypothesized that regulatory T cells are essential to the acquisition and maintenance of self-tolerance.

Type 1 diabetes is an increasingly common autoimmune condition, with 30,000 new diagnoses each year (JDRF Fact Sheet). Pancreatic islet transplantation holds great promise as a potential cure for this difficult disease; however human trials have had limited success. Attempts to promote self-tolerance or maintain a physical barrier to the transplanted islets have largely failed (Groot et al., 2004). Because of this, insulin dependence normally resumes five years post-operation. The deleterious effects of long-
term immunosuppression to promote extended islet survival are considered too great to justify this treatment.

Because of their important role in promoting self-tolerance, many immunologists believe regulatory T cells are the key to developing tolerance of islet allograft tissue. Rapamycin and anti-CD154 are immunoregulatory treatments that specifically inhibit the activation of T effector cells and promote the growth of regulatory T cell populations. As regulatory T cell numbers increase, self-tolerance is established and the need for immunosuppressant drugs is eliminated.

Unfortunately, treatments such as anti-CD154 and rapamycin have had limited success due to the ability of toll-like receptor (TLR) pathways to bypass such activation blockades. TLR stimulation results in a potent and direct activation that acts to bolster the immune response. This TLR activation results in the release of inflammatory cytokines, which render regulatory T cells unstable. Regulatory T cells have been shown to adopt effector phenotypes in such environments and may have pathogenic potential.

This study aims to elucidate aspects of Treg plasticity that result from TLR activation. *In vitro* models were used to demonstrate how TLR agonists change Treg phenotypic expression. Our findings indicate that the presence of lipopolysaccharides (LPS) has a relatively significant effect on regulatory T cell phenotypes. Specifically, our findings indicate that LPS causes increased GATA3 expression in Tregs, promoting differentiation to a T\(_{H2}\) phenotype \(p = 0.0543\). Regulatory T cells were also examined for the expression of ROR\(\gamma\)t and Tbet transcription factors. Neither transcription factor was significantly expressed, indicating the absence of T\(_{H17}\) and T\(_{H1}\) phenotypes,
respectively. It is also worth noting that stability of the foxp3 transcript appeared to be greater in cells treated with LPS, than in those without ($p=0.0009$).

In addition, this study utilized an *in vivo* model for tracking regulatory T cell changes after pancreatic islet transplantation. Diabetic reporter mice received pancreatic islet transplants, as well as TLR agonist to induce allograft rejection. Mice were treated with rapamycin, anti-CD154 and TLR agonist. After 12 days, regulatory and ex-regulatory T cells were harvested from the transplanted area and analyzed. This experiment is still in progress and results have yet to be determined.

This study establishes proof of concept of an effective system for the study of regulatory T cell plasticity. Additional investigation must be done in order to more thoroughly understand these important cells. This study is not complete, but our progress thus far is a strong foundation for further experimentation.
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<tr>
<td>ABM</td>
<td>Boston University</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen Presenting Cell</td>
</tr>
<tr>
<td>APC</td>
<td>Allophycocyanin</td>
</tr>
<tr>
<td>CD3</td>
<td>Cluster of Differentiation 3</td>
</tr>
<tr>
<td>CD4</td>
<td>Cluster of Differentiation 4</td>
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<tr>
<td>CD8</td>
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</tr>
<tr>
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</tr>
<tr>
<td>CD154</td>
<td>Cluster of Differentiation 154</td>
</tr>
<tr>
<td>CpG</td>
<td>Cystine-Phosphate-Guanine Oligodeoxynucleotides</td>
</tr>
<tr>
<td>CTLA4</td>
<td>Cytotoxic T-Lymphocyte-Associated Protein 4</td>
</tr>
<tr>
<td>DAMPs</td>
<td>Damage Associated Molecular Patterns</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
</tbody>
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DsRed.......................................................... Discosoma Sp. Red
ExRegs .......................................................... Ex-regulatory T cells
FBS ............................................................... Fetal Bovine Serum
FITC ................................................................. Fluorescein Isothiocyanate
FoxP3 .............................................................. Forkhead Box P3
GAPDH .......................................................... Glyceraldehyde 3-Phosphate Dehydrogenase
GATA3 ............................................................ GATA Binding Protein 3
GFP ................................................................. Green Fluorescent Protein
IACUC .............................................................. Institution of Animal Care and Use Committee
IFN-1 ............................................................... Type 1 Interferon
IFN-γ .............................................................. Interferon Gamma
IgG1 ................................................................. Human Immunoglobulin 1
IKK ................................................................. IκB Kinase
IL-1 ................................................................. Interleukin 1
IL-2 ................................................................. Interleukin 2
IL-4 ................................................................. Interleukin 4
IL-6 ................................................................. Interleukin 6
IL-7 ................................................................. Interleukin 7
IL-9 ................................................................. Interleukin 9
IL-10 .............................................................. Interleukin 10
IL-12 .............................................................. Interleukin 12
IL-15 .............................................................. Interleukin 15
IL-17 ................................................................. Interleukin 17
IMDM ............................................................. Iscove's Modified Dulbecco's Medium
IRAK ............................................................... Interleukin-1 Receptor-Associated Kinase
IRAK4 .............................................................. Interleukin-1 Receptor-Associated Kinase 4
IRF3 ................................................................. Interferon Regulatory Factor 3
IRF4 ................................................................. Interferon Regulatory Factor 4
LPS ................................................................. Lipopolysaccharides
MHC ................................................................. Major Histocompatibility Complex
MHCII .............................................................. Major Histocompatibility Complex II
mTOR .............................................................. Mammalian Target of Rapamycin
MyD88 ............................................................. Boston University
NF-kB ............................................................. Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells
PAMPS ........................................................... Pathogen Associated Molecular Patterns
PBS ................................................................. Phosphate Buffered Saline
PCR ................................................................. Polymerase Chain Reaction
PECy7 ............................................................. Phycoerythrin Cyanine-7
Post-op ............................................................ Post-operative
PTK ................................................................. Protein Tyrosine Kinase
RAPA .............................................................. Rapamycin
RFP ................................................................. Boston University
RNA ............................................................... Ribonucleic Acid
RORγt ............................................................. Retinoic Acid-Related Orphan Receptor Gamma
ROSA26 ................................................................. ROSAβgeo26 Locus
STAT3 ................................................................. Signal Transducer and Activator of Transcription 3
STZ ................................................................. Streptozotocin
T1D ................................................................. Type 1 Diabetes
Tbet ................................................................. T-box transcription factor TBX21
TBK ................................................................. Serine/Threonine-Protein Kinase
TCR ................................................................. T Cell Receptor
TGF-β ................................................................. Transforming Growth Factor β
tH1 ................................................................. T Helper Cell 1
tH2 ................................................................. T Helper Cell 2
tH17 ................................................................. T Helper Cell 17
TLR ................................................................. Toll-like Receptor
TLR3 ................................................................. Toll-like Receptor 3
TLR4 ................................................................. Toll-like Receptor 4
TLR7 ................................................................. Toll-like Receptor 7
TLR8 ................................................................. Toll-like Receptor 8
TLR9 ................................................................. Toll-like Receptor 9
TRAF ................................................................. TNF Receptor Associated Factors
TRAM ................................................................. TRIF-Related Adapter Molecule
Treg ................................................................. Regulatory T Cell
TRIF ................................................................. TIR-domain-containing adapter-inducing interferon-β
INTRODUCTION

A fundamental function of the body’s immune system is to identify and destroy foreign matter that has pathogenic potential. To accomplish this, the human immune system relies on a complex array of signaling pathways to identify and destroy harmful matter. Under certain circumstances, the immune system can become hypersensitive to healthy tissue. This leads to the unwanted destruction of necessary tissue, resulting in disease. This is the case in autoimmunity and transplant rejection. Under normal conditions, the immune system is kept in check by regulatory T cells (Tregs). Regulatory T cells play the pivotal role of down-regulating effector immune cell activity in order to prevent the destruction of healthy tissue. Cases in which the ratio of effector T cells to regulatory T cells is higher than normal have been shown to be associated with states of autoimmunity (Zhou et al., 2009; Bailey-Bucktrout et al., 2013). Furthermore, it has been well documented that the same phenomenon is found in tissue associated with transplant rejection. In contrast, when regulatory T cell are present in large quantities, immunosuppression and self-tolerance is observed (Sanchez-Fueyo et al., 2006). Regulatory T cells therefore help to maintain self-tolerance and prevent states of autoimmunity.

Regulatory T Cells

While working at Yale University in the late1960’s, Dr. Richard Gershon identified what he believed to be a subset of T cells that exhibited immunosuppressive
characteristics (Shevach et al., 2011). These T suppressor cells failed to gain acceptance due to an inability to positively indentify their distinct population. The scientific community gradually lost interest in T suppressor cells until the early 1990’s when a distinct population of T cells was identified that produced large amounts of the immunosuppressive cytokine, interleukin 10 (IL-10) (Choileain et al., 2006). These cells were identified as CD4+CD25+ lymphocytes. It was not until Sakaguchi and his colleagues identified FoxP3 as an additional marker that regulatory T cells were accepted as a distinct T cell lineage (Choileain et al., 2006).

It has been demonstrated that a lack of CD4+CD25+ T cells leads to the onset of many autoimmune states, including type 1 diabetes, chronic gastritis, and rheumatoid arthritis (Hori et al., 2003; Sakaguchi et al., 1995; Choileain et al., 2006). In 2003 Hori et al were able to show that retroviral gene transfer of Foxp3 converted naïve T cells to a regulatory phenotype (Hori et al., 2003). Based on their data, Hori and colleagues hypothesized that Foxp3 is a key regulatory gene for the production and development of regulatory T cells. For years, immunologists have been hopeful that they might be able to use regulatory T cells as a way of naturally altering an individual’s immune system to favor a non-active state. Most of these efforts have failed due to the instability of regulatory T cells in vivo.

The Danger Hypothesis

It has been well established that injection of a foreign antigen does not always promote an immune response. To explain this fact, it was hypothesized that our bodies
have ways of determining which foreign material has pathogenic potential and should be eliminated. In the 1920’s, Ramon and Glenny observed that a significant immune response is only observed in the presence of certain molecular patterns, which they called adjuvants (Kono et al., 2008). For decades, the question as to why these adjuvants were so effective went largely unexplored. It was not until the 1980’s that immunologists began to theorize how the immune system determines which foreign antigens present a danger to the body. In 1989, Janeway postulated that the APCs of the innate immune system recognize specific molecular patterns that have pathogenic potential (Kono et al., 2008). Janeway named these activation initiators pathogen-associated molecular patterns (PAMPs) and identified non-mammalian microorganisms as their source.

Subsequent experimentation has shown that Janeway’s hypothesis is largely correct; however, it fails to explain why primary and secondary cell necrosis triggers an immune response. Specifically, it did not account for the inflammation seen in post-operative patients (i.e.: transplantation). In 1994, Matzinger formulated a hypothesis meant to explain inflammation driven by non-apoptotic cell death (Kono et al., 2008). According to her hypothesis, during necrotic cell death, damage-associated molecular patterns (DAMPs) are released into the blood stream and trigger an immune response. Matzinger postulated that DAMPs act as a universal danger signal and can change the state of our immune system from stable to active.
Matzinger’s danger hypothesis has since been validated and it is suspected to be a major reason that regulatory T cell therapy is not yet reliable to prevent transplant rejection. Even if donor and recipient are well matched, transplant rejection may occur due to cell death and the release of DAMPs after transplantation. The presence of DAMPs signals to the immune system that something is amiss. Cells of the immune system subsequently adopt the activated phenotype and have the unwanted effect of
destroying the allograft tissue. This is similar to an autoimmune state, in which there is an imbalance between regulatory immune cells and those that are in the active immune state.

*Implications of the Danger Hypothesis on Regulatory T Cell Phenotype*

Komatsu et al have shown that regulatory T cells differentiate into T\(_{H17}\) cells in arthritic conditions (Komatsu et al., 2014). Regulatory T cells that have differentiated to adopt an effector phenotype are broadly considered ex-regulatory T cells, or ExRegs. By having effector functionality, ex-regulatory T cells may contribute to the pathogenesis of the autoimmune condition. This finding suggests that under non-pathogenic conditions, there is a balance between the number of effector and regulatory T cells. If that balance shifts to favor the presence of relatively more effector T cells, then autoimmunity and transplant rejection occur. It has been postulated that blocking specific pathways necessary for the transition from a regulatory to effector phenotype would greatly increase the probability of successful tissue transplantation. In 2005, Peng and colleagues were able to show that activation of toll-like receptor 8 reversed regulatory T cell function, and promotes the effector phenotype (Peng et al., 2005).
Figure 2. Foxp3+ T cell differentiation into effector T cell phenotypes. Various inflammatory conditions have been shown to be associated with specific changes in Treg phenotypes. (Figure taken from Cretney et al., 2013)

Furthermore, Peng concluded that regulatory T cell transdifferentiation is dependent on a functional TLR8-MyD88-IRAK4 signaling pathway in antigen presenting cells. In T cells, transdifferentiation has been shown to be dependent on several distinct transcription factors that control gene expression. For example, retinoic acid-related orphan receptor- γt (RORγt), T-bet, and GATA-binding protein-3 (GATA3) are known to be associated with controlling T cell function (Campbell et al., 2011). Activation of these transcription factors leads to the expression of the T17, T1, and T12 cell phenotype, respectively. Conversely, transcription factors that block Treg differentiation have been identified as well. Examples include the signal transducer and activator of transcription 3 (STAT3) and the interferon regulatory factor 4 (IRF4) transcription factors. These
transcription factors block the differentiation of Treg cells into $T_{H17}$ and $T_{H2}$ cells, respectively. These findings indicate that manipulating these pathways with small molecule ligands may have the potential to increase regulatory T cell stability and open the door for the possibility of regulatory T cell therapies.

Figure 3. Transdifferentiation of regulatory T cells into effector T cell phenotypes is blocked by STAT-3 and IRF-4 dependent mechanisms. Regulatory T cells functionality is maintained by various transcription factors. For example, STAT-3-dependent mechanisms block transdifferentiation of Tregs into $T_{H17}$ cells, while IRF-4-dependent mechanisms block transdifferentiation into $T_{H2}$ cells. Conversely, GATA3, RORγt, and T-bet transcription factors promote the conversion of Tregs into $T_{H2}$, $T_{H17}$, and $T_{H1}$ cells, respectively. (Figure taken from Campbell et al., 2011)

Several cytokines released by antigen presenting cells (ie.: dendritic cells) have been identified in playing key roles in T cell transdifferentiation. Upon activation, T cells may adopt a number of different phenotypes, depending on the cytokines produced and where in the body the Treg is located. For instance, in the case of infection, the presence of TGF-β and Interleukin 6 will promote the expression of $T_{H17}$ phenotypic expression (Campbell et al., 2011). On the other hand, IFNγ and interleukin 12 will direct a Treg to
differentiate into a $T_H1$ effector T cell (Kohno et al., 1997). And finally, interleukin 4 is a potent inducer of the $T_H2$ phenotype (Swain et al., 2011).

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>Phenotype Expressed</th>
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<tr>
<td>TGF-$\beta$ + IL-6</td>
<td>$T_H17$</td>
</tr>
<tr>
<td>IFN-$\gamma$ + IL-12</td>
<td>$T_H1$</td>
</tr>
<tr>
<td>IL-4</td>
<td>$T_H2$</td>
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Table 1. Different inflammatory environments promote the conformational change of regulatory T cells to effector phenotypes. ExRegs are made when regulatory T cells are exposed to an inflammatory environment. The new phenotypic identity of ExRegs is dependent on the cytokines present and the type of tissue where the Tregs are located. (Campbell et al., 2011)(Swain et al., 2011)(Kohno et al., 1997)

In order for the phenotypic changes discussed above to occur naturally on a large scale, APC induced T cell activation must occur. The mechanism for this activation occurs in a stepwise fashion. The first step involves the classic model of T cell receptor (TCR) activation by the major histocompatibility complex (MHC) of an antigen presenting cell. At this point, T cell activation is relatively weak and further costimulation is necessary for a robust response. Binding of a CD154 surface protein on a T cell to its complementary CD40 ligand on the APC marks the completion of step two. CD154-CD40 binding results in a large increase in the expression of CD80 and CD86 surface proteins on the antigen presenting cell. CD80 will then work in concert with CD86 to bind to the CD28 surface protein on a T cell. The APC will begin to produce and release an array of cytokines that cause phenotypic changes in the now activated T cell (T. Thornley, personal communication).
The three steps discussed above are generally needed for T cell activation. The exception occurs through direct activation via a toll-like receptor (TLR) agonist (Chen et al., 2006; Porrett et al., 2008). This has been of particular importance when attempting to induce tolerance by using blocking agents to prevent T effector cell activation. Therapies that utilize such mechanisms have had limited success due to the ability of TLR pathways to bypass CD40 activation and directly induce CD80/CD86 stimulation. Toll-like receptors bind to immunoreactive agents, such as lipopolysaccharides (LPS) and CpG oligodeoxynucleotides (CpG), and cause activation of downstream pathways leading to the production and release of cytokines (T. Thornley, personal communication).

There are several toll-like receptor pathways that are worth mentioning. Most TLR pathways can be divided into two groups: MyD88-dependent and MyD88-independent pathways. The MyD88-dependent pathway can associate with any TLR, except for TLR4, and is necessary for the production of inflammatory cytokines (Kaisho et al., 2006).

MyD88 pathways generally require the recruitment of IL-1 receptor associated kinases (IRAK), which associate with TRAF and IKK, leading to the activation of the NF-κB transcription factor. NF-κB activation is associated with effector T cell phenotypes and leads to the production of several inflammatory cytokines. Alternatively, the MyD88-TRIF-IKK/TBK pathway used by TLR 4 activates the IRF3 transcription factor, resulting in the production of interferon-β (IFN-β) (Kaisho et al., 2006). IFN-β is
a cytokine that induces either immunoactivation or suppression, depending on the environment in which it is found (T. Thornley, personal communication).

The second TLR pathway subset, the MyD88-independent pathway, is primarily involved in signaling for the production of interferon cytokines. The two most notable TLRs that utilize this pathway are TLR3 and TLR4. TLR3 associates directly with TRIF and ultimately induces activation of IRF3. TLR 4 also utilizes this TRIF-dependent pathway; however, it must first interact with a TRIF-related adapter molecule (TRAM) in order to do so (Kaisho et al., 2006).

Toll-like receptors have been shown to have different type ligands depending on their positioning in the cell. For instance, toll-like receptors located in endosomes interact with single and double stranded DNA and RNA. These toll-like receptors, which include TLR3, TLR7, and TLR9, are typically involved in the immune response to viral infection and the presence of foreign DNA. CpG oligodeoxynucleotide (CpG), for example, is a single stranded DNA fragment that activates TLR3 and leads to the production of inflammatory cytokines. Toll-like receptors located on the cell surface generally ligate with proteins associated with bacteria (PAMPs) and necrotic debris (DAMPs). Toll-like receptors 1, 2, 4, 5, and 6 are several of those that fall under this category (Kaisho et al., 2006). Lipopolysaccharides, an important protein associated with gram-negative bacteria, interacts with TLR4 and activates a NFκB transcription factor, leading to the production of inflammatory cytokines (Kaisho et al., 2006; T. Thornley, personal communication).
As mentioned earlier, activation of toll-like receptors results in pathways that bypass the three steps needed for T cell activation. LPS and CpG both utilize these bypass pathways and result in the production of inflammatory, and potentially pathogenic cytokines. For example, TLRs 3, 4, 7 and 9 will upregulate CD80/CD86 expression, bypassing the need for CD28-induced activation (T. Thornley, personal communication). Alternatively, activation of TLRs 1, 2, 4, 5 or 6 will lead to NFκB expression and directly induces the production of inflammatory cytokines. The implications that these bypass pathways have on the development of a drug to induce transplant tolerance are immense. Several therapies that are either currently used, or are being evaluated for potential use are worth mentioning.

**Current/Potential transplant therapies**

**Anti-CD154**

CD154 is expressed on a variety of cell types (T cells, macrophages, antigen presenting cells, etc) and plays a major roll in the immunoactivation of T cells (Kirk et al., 2001). The three-step model mentioned earlier mediates T cell activation. According to this model, three steps are required for effective T cell activation. The first is generated from the binding of the T cell receptor to the major histocompatibility complex (MHC) ligand. Signal two is generated upon binding of CD154 on the T cell to its ligand, CD40, on an APC (Kirk et al., 2001). Binding of a T cell’s CD28 surface protein to its CD80/CD86 complement on the APC leads to the initiation of signal three, which causes the production and release of inflammatory cytokines (T. Thornley, personal communication).
communication). When the TCR, CD154 and CD28 complexes are bound to their respective ligands, the T cell and APC are most active and will undergo processes to create a more robust immune response. Specifically, ligation of CD40 has been shown to upregulate expression of MHC II on antigen presenting cells, thereby enhancing their ability to interact with T cells (Kirk et al., 2001). Additional studies by this group have shown that CD154 binding increases expression of costimulatory molecules CD80 and CD86 on T cells. This increased expression promotes the ability of CD86 and CD80 to bind to its CD28 complement, which dramatically increases cytokine release. Anti-CD154 is effective in blocking the binding and activation of the CD154 complex; thereby suppressing the immune response generated by APC-T cell binding. Specifically, anti-CD154 blockade prevents signal three from occurring by inhibiting the upregulation of CD80/CD86 transcription factors (Porrett et al. 2008; Chen et al. 2006). In addition to blocking effector T cell activation, CD154 blockade has been associated with regulatory T cell expansion and the induction of permanent tolerance (Rigby et al., 2008). Although anti-CD154 has demonstrated enormous potential in both mouse and monkey models, it is currently not used in humans due to significant side effects.
Figure 4. Treatment with Anti-CD154 induces tolerance. A) The three-step model for T cell activation. TCR binding to MHC complex leads to increased expression of the CD154 surface molecule. CD154 then binds to CD40 on an APC, resulting in increased expression of the CD80/CD86 surface protein complex. This upregulation enables increased binding to the CD28 costimulatory molecule and T cell activation. B) Anti-CD154 induces tolerance by blocking CD40-CD154 interaction. This blockade prevents upregulation of the CD80/CD86 complex, inhibiting costimulation and T cell activation. (Figure taken from T. Thornley, 2006)
**Rapamycin**

Rapamycin (RAPA) is an immunosuppressant commonly used in patients after kidney transplant due to its low toxicity towards the kidneys. Rapamycin acts as an immunosuppressant by blocking IL-2, IL-4, IL-7, IL-12, and IL-15-driven T cell proliferation (Sehgal, 1998). RAPA is also known to stop the cell cycle in the mid to late G\textsubscript{1} phase (Sehgal, 1998). RAPA is the inhibitor of the mammalian target of rapamycin pathway (mTOR), thereby preventing T cell activation (Foster et al., 2010).

**Belatacept and Abatacept**

Belatacept is a treatment designed to block the interaction of CD28 of the T cell with its CD80/CD86 ligand on an APC. It accomplishes this through its very high affinity for the CD80/CD86 complex, thereby preventing the CD28 surface protein from gaining access to its compliment. Blockage of the CD80/CD86 complex prevents both the initiation of signal three, and the promotion of inflammatory cytokine release. This lack of stimulation results in T cell anergy and eventual apoptosis (Vincenti et al., 2005; Vincenti et al., 2010).

Similar in structure to Belatacept, Abatacept also inhibits T cell activation via binding to CD80 and CD86. Abatacept is a fusion protein made from the extracellular portion of cytotoxic T lymphocyte-associated antigen 4 (CTLA4) and the constant region fragment of human immunoglobulin 1 (IgG1). CTLA4 is a surface protein found on T cells that binds to an APC and acts to downregulate immunoactivation. Abatacept mimics
this property of CTLA4 and promotes immunosuppression. Abatacept is highly efficacious in treating several autoimmune diseases, including rheumatoid arthritis and psoriasis. Abatacept, however, has had less success in promoting the acceptance of transplanted tissue. Belatacept is considered a much strong immunoregulator and has been much more successful in the transplant model (Genovese, 2005; Vincenti et al., 2005).

Successful transplantation is of pivotal importance in combating some of the most prevalent diseases. Type 1 diabetes (T1D), for example, affects 30 million Americans, with 30,000 new patients diagnosed each year (JDRF Fact Sheet). Islet Transplantation has been shown to be very effective in reversing T1D, with individuals remaining insulin independent for at least one year post-operation. Islet or whole pancreas transplantation is the closest treatment to a cure for T1D; however, these methods are seldom used clinically. Attempts to promote self-tolerance or maintain a physical barrier to the transplanted islets have largely failed (Groot et al., 2004). Because of this, insulin dependence normally resumes fiver years post-operation. The deleterious effects of long-term immunosuppression to promote extended islet survival are considered too great to justify this treatment.

Regulatory T cell therapies have great potential to maintain immunosuppression without compromising the health of the recipient. It has been well documented that Tregs are very effective in promoting immunosuppression, and are crucial to maintaining self-tolerance. Several therapies, such as Belatacept and Anti-CD154, which are currently
used in humans or monkeys, have shown great potential in expanding regulatory T cell populations and promoting long-term tolerance. However, success with these drugs has been limited due to TLR activation and subsequent bypass of the T cell-stimulation blockade (Porrett et al., 2008). Continued investigation of these TLR pathways and their resulting cytokines is therefore of upmost importance.

Reliable regulatory T cell expansion and the induction of tolerance has breakthrough potential in transplantation. Regulatory T cell therapies have further potential to correct states of autoimmunity. It is therefore of great importance that further efforts be made to better understand these cells. The goal of this research thesis is to elucidate how regulatory T cell phenotypes change in several different models of islet transplantation. Specifically, this project uses islet transplantation in mouse models to determine what types of regulatory T cell-derived phenotypes are expressed after transplant rejection. In addition, this study will investigate how TLR agonists affect regulatory T cell function and phenotype. ExRegs will be analyzed for which transcription factors are expressed, and which signaling molecules these cells may be producing.

**Specific Aims/Objectives**

Regulatory T cells are known to have a great amount of plasticity in vivo. Treg phenotypes have been observed to change from having immunosuppressive to effector roles under certain conditions; however, regulatory T cell plasticity has yet to be thoroughly examined in pancreatic islet transplant models. The differentiation of Tregs
into effector T cells likely has major implications to the survival of allograft tissue. Therefore, the function of regulatory T cells in transplantation models has great clinical relevance and should be further investigated.

The goal of the present study is to determine how regulatory T cell phenotype’s change after transplantation. Specifically,

(1) What other T cell phenotypes (if any) are expressed in transplant tissue? The goal here is to determine what markers these cells express. For example T₇, T₂, T₁, etc.

(2) *In vitro* studies will be conducted as well to identify any differences from the *in vivo* models that might be present. Consistencies between the *in vivo* and *in vitro* studies will strengthen to the results of this study.

(3) Functional assays will be performed to determine that these ExRegs behave as their respective phenotypes do.

a. What cytokines are ExReg cells producing, and how much of each? For example, do ExReg T₁7 cells produce the same signaling molecules (ie.: IL-17) in the same quantity as those T₁7 that are not ExRegs?

b. This study will examine CD4⁺Foxp³⁺ T cells after exposure to PAMPs to determine if these cells still express the regulatory phenotype. We postulate that Foxp3 may still be expressed on the cells surface while transcription for this protein has been shut off. Therefore, the cell expresses the regulatory T cell phenotype while functionality may be similar to something in between a Treg and effector T cell.
(4) We hope to identify signaling pathways within regulatory T cells that are necessary for their conformational change into ExReg. By identifying these mechanisms, our hope is that small molecule ligands might be identified that have the potential to block the Treg to ExReg change.

This study’s purpose is to elucidate aspects of regulatory T cell plasticity in transplanted mouse models. The overarching goal of which is to provide information that might be useful in developing post-operative transplant therapies. Regulatory T cell therapies have the potential to dramatically increase rates of successful transplantation without the use of long-term immunosuppressive drugs. By doing so, transplantation will be more reliable, and the risk of complication will decrease.
METHODS

The methods and materials used for this study were based on those proposed in a grant submitted to the National Institute of Health and the American Diabetes Association. Mice used were bred and stored in Beth Israel Deaconess Medical Center’s pathogen-free barrier facility. All regulatory guidelines set forth by Beth Israel Deaconess Medical Center and the Institution of Animal Care and Use Committee (IACUC) were strictly followed.

In Vitro Experiments

Mixed Lymphocyte Reactions (MLR) were used to examine regulatory T cell responses in vitro. Two mice were sacrificed to carry out each of these experiments. One mouse served as the regulatory and effector T cell donor, while the other provided antigen presenting cells (APCs). Auxiliary, brachial and mesenteric lymph nodes, as well as the spleen were excised from the T cell donor. Only the spleen was taken from the APC donor mice. Tissues were homogenized and antibodies added for staining purposes.

MLR with GFP/RFP reporter mice

Foxp3.GFP-Cre.Rosa26.RFP (hereafter referred to as GFP/RFP) strain reporter mice are bred with knock-in green florescent protein (GFP) as a marker for gene expression of the foxp3 transcript. Red florescent protein (RFP) transgene is inserted into the Rosa26 locus and flanked by loxP sites. Mice that have both markers cannot express GFP without RFP. Therefore a mouse that is double positive for GFP and RFP expresses...
the foxp3 transcript. A mouse that is only positive for RFP expresses the foxp3 transcript in the past, but does not do so currently. Foxp3 is considered to be a crucial protein for the function of regulatory T cells (Bailey-Bucktrout, 2013). Therefore, cells that are GFP+RFP+ represent regulatory T cells, while GFP-RFP+ cells are ex-regulatory T cells. GFP-RFP- cells were never regulatory and are characterized as effector T cells.

Figure 5. Graphic representation of fluorescently labeled cell identities. Cells that are GFP+RFP+ are Tregs. Cells that are GFP-RFP+ are ExRegs. Cells that are GFP-/RFP- are T effector cells (original figure).

T cells are harvested from GFP/RFP reporter mice by excising the spleen and lymph nodes. Spleen and lymph nodes are homogenized, washed and filtered to remove impurities and fat that may have been present on the tissue surface. APC/Alexa Fluor-647 anti-CD4 antibody was used to stain T cells. Anti-CD4 antibody is widely used as a marker for T helper cells and is known to exist on the surface of regulatory T cells.

Antigen presenting cells were harvested from the spleens of either DBA/2J strain mice or non-expressing reporter mice. Spleens were homogenized, washed, and filtered to remove impurities and fat that may have been present on the tissue surface. Staining
protocol incorporated the use of ACP/Alexa Fluor-647 anti-CD11c antibody, as well as PECy7 anti-CD11b antibody.

After washing away excess antibody, 4',6-diamidino-2-phenylindole (DAPI) staining was applied to both antigen presenting cells and T cells. DAPI is a blue fluorescent dye that binds to adenine-thymine rich regions of DNA. Because DNA is generally only exposed during apoptotic and necrotic events, DAPI is used to label dead or dying cells.

After staining with DAPI, the cells are ready to be sorted. Cells were sorted using flow cytometric analysis and a Becton Dickinson FACSARia™ III sorter. The antigen presenting cells were gated DAPI negative, CD11c high, and CD11b intermediate/high and then sorted. Regulatory T cells were gated DAPI negative, CD4 high, GFP high and RFP high and sorted. T effector cells were gated DAPI negative, CD4 high, GFP low and RFP low. The presence of GFP and RFP was identified with the use of fluorescein isothiocyanate (FITC) and DsRed fluorochromes, respectively. Cells were sorted into T cell media for increased viability. T cell media was made from IMDM with 10% FBS and Penicillin-Streptomycin-L-glutamine.

Once the cells had been sorted, they were centrifuged and brought into a sterile hood for plating. T cells were resuspended at a final concentration of 1,000 cells per microliter. Final concentration of antigen presenting cells was one cell per 10 microliters. Cells were plated using 300 microliter round-bottom wells. One hundred thousand T cells were plated with ten thousand antigen-presenting cells. Interleukin 2 (IL-2) at a concentration of 20 ng/ml was added to each well to allow for increased regulatory T cell
viability. Regulatory T cells were exposed to two treatments: one with Lipopolysaccharides (LPS), and the other without. LPS was added at a concentration of 1 µg/ml. LPS is a large endotoxin found on the outer membrane of gram-negative bacteria, and used here to illicit an immune response.

An alternative method used in the study was the use of anti-CD3/anti CD28 co-stimulation. Anti-CD3 at a concentration of 2 µg/ml was used to coat flat-bottom well plates prior to addition of lymphocytes. After about four hours of anti-CD3 incubation, the wells were washed three times with phosphate buffered saline (PBS) and lymphocytes were added at the same concentration as stated above. In addition to IL-2 and LPS, anti-CD28 was added at a concentration of 4µg/ml. A combination of anti-CD3 and anti-CD28 antibodies has been shown to directly stimulate CD4+ and CD8+ T cell proliferation by mimicking the action of antigen presenting cells (Thomas et al., 2002). This protocol was used in the hopes of increasing T cell viability in vitro by inducing a more potent stimulation.

Plated cells were allowed to incubate and later analyzed at the day 5 time point. On day 5, cell were gently extracted from wells and placed in test tubes. The cells were then washed and resuspended in PBS with 1% fetal bovine serum (FBS). Anti-CD4 APC antibody and DAPI were added for staining. Cells were then subject to flow cytometric analysis and sorted. Sorted ex-regulatory T cells were found by gating on CD4 high, DAPI low, GFP low and RFP high. T effectors were also sorted by gating on CD4 high, DAPI low, and GFP and RFP low. Cells were sorted into lysis buffer and analyzed by single cell real-time PCR. The kit number used for the PCR is 46-7200. Cells were
analyzed for the presence of several transcription factors. GAPDH is a housekeeping
gene used here to confirm the presence of a cell in each well. Wells lacking GAPDH
were ignored. CD11C primer was added to eliminate dendritic cells from PCR analysis.
RORγt, Tbet, and GATA3 were used to identify effector phenotypes.

**MLR with transgenic ABM x GFP/RFP reporter mice**

In the initial mixed lymphocyte reactions of this study, regulatory T cell
stimulation by the DBA/2J APCs was relatively low. T cell receptor (TCR) specificity of
regulatory T cells is known to be relatively heterogenic; however, it was decided that this
study would benefit from a more specific system. T cells of the ABM TCR transgenic
mouse have a Vα2.1 and a Vβ8.1 T cell receptor specific for the I-A<sup>BM12</sup> molecule, and
will not respond to other alloantigen (Sanchez-Fueyo et al., 2006). The I-A<sup>BM12</sup> antigen is
expressed on the B6.C-H<sup>BM12</sup>/KhEg, hereafter referred to as bm12, mouse strain,
(Sanchez-Fueyo et al., 2006).

Breeding a Foxp3.GFP-Cre.Rosa26.RFP reporter mouse with an ABM TCR
transgenic mouse made it possible to harvest TCR specific regulatory T cells that had
reporter function. Breeding parental generations of these two mice theoretically results in
100% of the F1 generation expressing the ABM transcript, while 56.25% will express the
GFP/RFP reporter transcripts. In this model, T cells were taken from the GFP/RFP x
ABM TCR transgenic mice, while antigen presenting cells were harvested from the bm12
mouse strain. This model facilitates MHC class II specific binding between the ABM T
cell receptor and the I-A<sup>BM12</sup> antigen. This TCR specificity results in a much stronger
activation of regulatory T cell receptors, thereby increasing proliferation and viability of the cells of interest.

![Figure 6. Mixed lymphocyte reaction model with TCR transgenic mouse model.](image)

ABM TCR transgenic mice were bred with GFP/RFP reporter mice to obtain T cells with greater TCR-antigen specificity. Antigen presenting cells were harvested from the bm12 mouse strain. These mice contain an I\(^{-}\)A\(^{bm12}\) antigen that specifically binds to the ABM T cell receptor (Original figure).

A slightly different staining protocol was used in this model. The day 1 staining remained the same for both the T cell and APCs. V\(\beta\)8 APC antibody was added to the day 5 protocol in order to positively identify cells that were bound to the bm12 antigen. In this model, ex-regulatory T cells were found by gating DAPI low, CD4 high, V\(\beta\)8 high and sorting the GFP low and RFP high population. This same gating protocol was used to identify regulatory T cells, except the sorted population consisted of GFP and RFP high cells.

**In Vivo Experiments**

Mouse models were used to illustrate the response of regulatory T cells by transplanting pancreatic islet allograft tissue into GFP-RFP reporter mice. Foxp3.GFP-Cre.Rosa26.RFP strain mice were used as transplant recipients, while pancreatic islets were isolated from mature DBA/2J strain mice. Five days prior to transplantation,
GFP/RFP reporter mice were given intraperitoneal injections of Streptozotocin (STZ) at 0.01 ml per gram mouse. STZ is a toxin known to destroy insulin-producing beta cells of the pancreas. STZ was used in this model to induce symptoms of type 1 diabetes. This method made it possible to determine the success of the islet transplantation by monitoring the blood glucose levels of the animals after surgery.

Blood glucose monitoring began four days before the transplantation to insure that all mice had developed symptoms of T1D. Mice that had blood glucose levels above 200 mg/dl were considered diabetic. Mice that had blood glucose levels of 500 mg/dl or higher were given either saline or insulin to ensure that they were healthy enough to undergo transplantation.

Allograft recipients were subject to four treatments:

<table>
<thead>
<tr>
<th>Treatment Group 1</th>
<th>Rapamycin + Anti-CD154 + TLR Agonist</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment Group 2</td>
<td>Rapamycin + Anti-CD154</td>
</tr>
<tr>
<td>Treatment Group 3</td>
<td>TLR Agonist Only</td>
</tr>
<tr>
<td>Treatment Group 4</td>
<td>Untreated</td>
</tr>
</tbody>
</table>

Table 2. Allograft recipients were subject to four different treatments (Original table).

The combination of rapamycin and anti-CD154 has been shown to be a powerful short-term inhibitor of pancreatic islet allograft rejection in non-human primates (Koulmanda, 2006). The success of this combination has sparked great interest in the scientific community with hopes of its application in humans. This study uses a rapamycin with anti-CD154 combination in order to maximize clinical relevance.
Treatments began on the day of transplantation. Rapamycin was administered at a dose of 3 mg per kg body weight. 250 microliters of Anti-CD154 monoclonal antibody treatment was injected into each mouse. CpG and LPS were both injected at a dose of 50 micrograms per gram mouse. Rapamycin was injected subcutaneously, while all other injections were administered intraperitoneally.

Rapamycin, anti-CD154, LPS, and CpG were administered daily for two days after treatment to their assigned treatment groups. On days three to six post-transplantation, only rapamycin was administered. Mice were injected with an additional dose of rapamycin on day nine. Tissue harvesting and analysis occurred on day twelve post-transplantation. The kidney, and spleen were excised from the specimen for analysis. Single cell real-time PCR was used to determine phenotypic expression. Kit number 467200 was used for the PCR. Cells were analyzed for the presence of several transcription factors. As in the in vitro experiments, GAPDH was used to confirm the presence of a cell in each well. CD11c primer was added to eliminate dendritic cells from PCR analysis. RORγt, Tbet, and GATA3 were used to identify effector phenotypes.

**TLR Agonists**

Lipopolysaccharides (LPS) and CpG oligodeoxynucleotides (CpG) were used as TLR agonists in this model. As mentioned earlier, LPS is a large endotoxin found on the surface of gram-negative bacteria. LPS works by binding to a TLR4-CD14 receptor complex on the surface of an antigen presenting cell. This binding activates protein tyrosine kinases (PTK) and leads to the mobilization of the NF-κB transcription factor (Ziegler-Heitbrock, 1995). CpG indirectly stimulates T cell activation by inducing type 1
interferon (IFN-1) production by antigen presenting cells (Sun et al., 1998). Once released into the circulation, IFN-1 binds to the type 1 interferon receptor on T cells and induces activation.
RESULTS

In Vitro Experiments

MLR using ABM x GFP/RFP Reporter Mice

Mixed lymphocyte reactions with this model had limited success due to difficulties in sorting an adequate number of regulatory T cells. For reasons unknown, these mice were consistently smaller than those of the GFP/RFP reporter strain and therefore had fewer regulatory T cells per mouse. It was never possible to analyze these cells with PCR due to low proliferation and very poor viability. Because of a ubiquitous lack of stimulation, most cells were found to be dead or dying by the day 5 sort.

After spending several months breeding these mice and attempting to produce results, it was decided to abandon this model. Regulatory T cells only make up between about 5 to 10 percent of the total T cell population (Ahmadzadeh, 2015). This fact makes it relatively difficult to find and sort T cells in vitro. We felt that our best option was to continue by using the GFP/RFP strain model to maximize regulatory T cell numbers.

MLR using GFP/RFP Reporter Mice

After several variations in technique, mixed lymphocyte reactions were successfully cultured and analyzed using this model. Regulatory T cell numbers obtained from these mice were relatively low as well.

Figure 6 shows Regulatory T cell sort data for day 1. Dead cells were excluded by gating CD4 (APC) high and DAPI low. The cell population that was sorted is shown in
the bottom right panel. These are the regulatory T cells, characterized as being double positive for GFP (FITC) and RFP (DsRed). Final count was 367,000 regulatory T cells sorted.

Figure 7. Day 1 flow cytometry data for sorting regulatory T cells. Cells were gated for CD4+FITC+DsRed+ character and sorted (original figure).

Dendritic cells (DC) were identified using flow cytometric analysis and sorted. Dead cells were excluded by gating for DAPI negative. The final sorted population is shown in the bottom right panel. The boxed region represents the dendritic cell population, identified as showing high expression for CD11c (APC) and high or intermediate expression for CD11b (PECy7). 100,000 dendritic cells were sorted and diluted to a concentration of 100 cells per microliter.
Figure 8. Day 1 flow cytometry data for sorting dendritic cells. Cells were gated for DAPI-CD11c+CD11b int/+ and sorted (original figure).

Cells were plated so that each well received 10,000 DC’s and approximately 91,000 regulatory T cells per well. Each well, already coated with anti-CD3 antibody, then received anti-CD28 and IL-2. Two wells received lipopolysaccharide while the remaining two did not receive any TLR agonist. The cells were left to incubate for five days and then analyzed and sorted. Sort data for day 5 is shown below. Tregs were sorted from both treatment groups (LPS+ and LPS-). Visual examination of the wells after incubation, as well as sort data indicated large amounts of proliferation. ExReg population should be GFP- and RFP+. However, upon flow cytometric analysis, it was decided that the best course of action would be to sort the GFP+/-. 
population for single cell PCR. This decision was based on previous difficulties in sorting a proper cell count. There was also a lack of clear differentiation between the two populations. Cells were sorted directly into a 96 well plate at a rate of one cell per well.

Figure 9. Flow cytometric data for single cell sort conducted after 5 days of cell culture. Data shown above is from treatment group with LPS. Tregs and ExRegs were sorted directly into a 96 well plate for single cell PCR analysis. Cells were gated DAPI-CD4+GFP+-RFP+ and sorted. Gating was identical for both LPS+ and LPS- treatment groups (original figure).

**Single Cell PCR**

Cells were successfully sorted into a ninety-six well plate and pre-amplified for cDNA replication. Wells that had either no GAPDH expression or showed expression for CD11c were eliminated, leaving eighty-seven wells for analysis. The transcription factor RORyt was not expressed in any well, suggesting that none of these cells had T\textsubscript{H}17
phenotypic expression. The presence of Tbet was minimal, with only three wells expressing Tbet expression in the treatment group without LPS. \( \text{T}_{\text{H}2} \) phenotypic expression was therefore insignificant.

GATA3 expression was relatively high in both treatment groups. Twenty-five wells from the group with LPS expressed GATA3, while eighteen did not. In contrast, only sixteen wells from the group without LPS contained GATA3, whereas twenty-eight did not. A chi-squared statistical analysis gave a \( p \) value equal to 0.0543 (\( p = 0.0543 \)). This \( p \) value represents a likely significant difference in the number of wells expressing GATA3 in the treatment group with LPS, versus the group without. This finding suggests that the presence of lipopolysaccharide had a relatively significant impact in promoting the expression of the \( \text{T}_{\text{H}1} \) phenotype.

![GATA3 expression graph]

\[ p = 0.0541 \]

**Figure 10.** Graphic representation of the number of cells that exhibit expression of GATA3 from each treatment group. The difference in the number of wells expressing GATA3 between each treatment group approached significance. The treatment group with LPS had a significantly greater number of wells expressing GATA3 (original figure).
The amount of GATA3 expressed by each cell was then compared between treatment groups. Each GATA3 expressing cell was standardized against its GAPDH expression, and a logarithmic value of relative expression was generated. These values were then plotted and analyzed for statistical significance. Fischer’s exact test was used, which generated a p value of 0.5, suggesting that there was no significant difference in amount of expression between treatment groups. Although the group treated with LPS showed a greater number of cells expressing GATA3, the difference in expression between the two groups was insignificant.

![GATA3 expression](image)

**Figure 11.** Graphic depiction of the amount of GATA3 expression by cells between treatment groups. Although cells treated with LPS showed slightly higher expression of GATA3, the difference was not significant (original figure).

The expression of foxp3 was compared across treatment groups. The retention of Foxp3 expression was significantly greater in the treatment group with LPS. A chi squared statistical analysis of this finding generated a p value of 0.0009. In the treatment group with LPS, 34 cells retained expression of the foxp3 transcript, whereas just 9 cells
did not. In contrast, in the treatment group without LPS, 19 cells continued to express foxp3, while 25 did not.

![Figure 12. Retention of Foxp3 expression across treatment groups.](image)

A significantly greater number of Tregs in the treatment group with LPS retained foxp3 expression ($p=0.0009$). Cells in the treatment group without LPS tended to lose their foxp3 expression (original figure).

**In Vivo Experiment**

This study’s *in vivo* experiments are still in progress. Four mice were given injections of streptozotocin and subsequently developed type 1 diabetes. Blood glucose levels were monitored closely to confirm symptoms of T1D. As of this writing, we are seven days post islet transplantation. Successful transplantation has been indicated by the return of all specimens’ blood glucose levels to normal range 36 hours post operation.

This is the first round of *in vivo* experiments, so it was decided to start with just two of the four treatment groups. All four mice received Rapamycin and CD154. Two mice (one male, one female) were also given CpG and LPS as TLR agonists. This
correlates to treatments one and two, as stated previously in the methods section. TLR agonist was given to treatment group two on days zero (day of transplant) through two, as well as day five post operation. TLR agonist will also be administered on day 8 post-op, leading up to tissue harvest. Thus far, the mice are doing as expected and the experiment is progressing smoothly.
DISCUSSION

This project has established proof of principle by demonstrating a model that is effective for studying phenotypic changes in regulatory T cells (Tregs), both in vivo and in vitro. This is the first study of its kind to specifically examine the effect that toll-like receptor (TLR) agonists have in promoting allograft rejection via their effects on regulatory T cells. The beneficial effects that rapamycin and CD154 have in promoting allograft acceptance have been well established; however, investigators have demonstrated conclusively that TLR agonists bypass the CD154-Rapamycin T cell activation blockade and cause allograft rejection. This is the first study that aims to elucidate the mechanism by which this happens in a diabetic mouse model.

Regulatory T cells are historically difficult to study, mainly because of their minority presence and their instability in vitro. Most published studies that have elucidated much of what we know about regulatory T cells have come from in vivo models. This study demonstrates a successful and reliable protocol for studying Tregs in vitro. Interleukin-2 (IL-2) has been described as a cytokine necessary for Treg stability and long-term survival. We confirm that the addition of IL-2 results in much greater viability, enabling more dependable flow cytometric analysis and an increased cell count for sorting.

The choice to use single cell PCR, instead of pooled PCR, allowed for greater consistency in producing results. Our pooled real-time PCR protocol requires a minimum
of two hundred and fifty cells for reliable results. After incubation, staining, and lysis, that number proved difficult to obtain from one mouse. Single cell PCR, although more expensive, allows investigators to use just one mouse as a Treg donor and produces more consistent results.

As previously stated, our ABM x GFP/RFP mouse strain proved unsuccessful in producing results. Our hope was that the ABM-bm12 antigen specificity would result in increased proliferation, enabling the use of pooled real-time PCR; however, the low regulatory T cell number per mouse made this a challenge. We hypothesize that inheritance of the desired transgene led to the development of mice with stunted growth and less than a usable number of Tregs. We predict that using two mice as Treg donors, instead of one, may resolve this problem; however, the time required for breeding made this option impractical.

This in vitro study using GFP/RFP reporter mice produced results using a consistently reproducible model. The results obtained from single cell PCR indicate a greater number of regulatory T cells expressing an effector phenotype in those treated with lipopolysaccharide (LPS). Specifically, the number of Tregs expressing GATA3 was higher in the LPS treatment group, compared to the group without LPS. This result indicates that the presence of LPS likely caused regulatory T cells to adopt a T_{H2} effector phenotype. As previously discussed, TLR agonists act on antigen presenting cells to directly upregulate expression of CD80/CD86 costimulatory molecules. In doing so, TLR agonists effectively bypass immunosuppressant drugs such as rapamycin or anti-CD154. The exact mechanism of regulatory T cell transdifferentiation into the T_{H2} phenotype by
way of TLR agonist is unclear. It is also yet to be determined whether T_{H2} will be the dominating phenotype in the *in vivo* model.

There was no significant difference in relative expression of GATA3 by cells across treatment groups. In other words, T_{H2} cells in the treatment group with LPS did not differ significantly in their amount of GATA3 expression from those that were not treated with LPS. This insignificance was not surprising and simply demonstrates that T_{H2} cells may have a basal amount of GATA3 expression, regardless of their environment. Of greater importance was the finding that LPS seems to trigger a response from Tregs, resulting in their transdifferentiation into a T_{H2} cell.

It has been well established that a major role of T_{H2} cells is the production of interleukin 4 (IL-4). Through a positive feedback mechanism, this IL-4 production leads to greater T_{H2} proliferation (Brown, 2008). IL-4 has many functions that result in a more robust inflammatory response. One such function is the induction of monocyte-derived macrophages from the blood. In the presence of IL-4, these macrophages have been shown to proliferate and become active, leading to potential pathogenesis (Jenkins et al., 2011). It has also been demonstrated that the presence of IL-4 prevents the formation of TGF-β-derived regulatory T cells. Instead, IL-4 induces proliferation of IL-9 and IL-10 producing T effector cells, resulting in an even more robust inflammatory response (Dardalhon et al., 2009). Therefore, although T_{H2} by itself is a relatively benign inflammatory agonist, its presence indicates the possibility of slow-onset inflammation. *In vivo*, for example, this might result in chronic allograft rejection.
No RORγt expression was seen in any wells and an insignificant presence of Tbet was observed in three wells of the treatment group lacking LPS. This finding indicates that TLR-induced transdifferentiation of Tregs did not result in the appearance of T_{H1} or T_{H17} phenotypes. It is well established that TGF-β induces naïve T cells to adopt a regulatory phenotype. It is also known that TGF-β with the addition of IL-6 promotes the formation of highly potent T_{H17} inflammatory cells (Bettelli et al., 2006). Because of this common lineage, questions have been raised as to whether regulatory T cells have the ability to adopt a T_{H17} phenotype in certain immuno-environments. This in vitro model suggests that these lineages are distinct within the tested environment.

An unexpected loss of foxp3 expression by regulatory T cells in the treatment group without LPS was observed. The foxp3 transcript is typical of the regulatory phenotype, and is usually lost upon differentiation. Furthermore, foxp3 expression has been shown to become unstable in pathogenic environments (Zhou et al., 2009). Therefore, our finding that regulatory T cells treated with LPS seemed to have more stable expression of foxp3 than in those without LPS was unexpected. This finding indicates that the loss of foxp3 expression is not necessary for regulatory T cells to adopt the T_{H2} phenotype.

As stated previously, in vivo models for studying regulatory T cells are more reliable, and more accurately depict the natural immunophysiology of regulatory T cells. For this reason, moving forward the bulk of this project’s focus will be towards in vivo testing and results. Our in vivo models have progressed nicely and, as of this writing, are expected to produce results within the next week. The in vitro and in vivo models
discussed in this paper represent the groundwork for future testing. Several additional in vivo and in vitro experiments will be conducted to further this investigation.

Single cell PCR panels will be expanded to include additional transcription factors and cytokines. Transcription factors may be added to the panel based on previous findings. For example, presence of IL-4 will be examined to determine whether GATA3 expressing cells behave as natural T\textsubscript{H}2 cells. Future studies will utilize a larger cohort of animals in order to strengthen our statistical analyses. The focus of the experiments will gradually shift from in vitro to in vivo models in order to produce results consistent with a regulatory T cell’s natural environment. Additional analyses will include a comparison of regulatory T cell phenotypes to those expressed by T effectors. T effector transcription factor expression and production of cytokines will be compared to differentiated Tregs that are phenotypically similar. This type of analysis will be used to determine if, for instance, Treg-derived T\textsubscript{H}2 cells produce similar quantities of inflammatory cytokine as T\textsubscript{H}2 cells that were sorted from the T effector population.

**Conclusion**

In healthy individuals, the immune system is a carefully orchestrated balance between activation and suppression. Detriments to health associated with immunodeficiency and autoimmunity evidence the critical importance of maintaining this balance. As potent suppressors of immunoactivation, regulatory T cells play a critical role in preventing hyperactivity of our immune system. Examples of such hyperactivity include autoimmunity and allograft rejection. It has been demonstrated that maintaining
high numbers of regulatory T cells results in allograft tolerance. Furthermore, a common characteristic of autoimmunity is an imbalance between T effector and regulatory T cells, favoring the former. Therefore, regulatory T cells play pivotal roles in both the establishment and maintenance of self-tolerance.

Despite major advances in the scientific community’s understanding of regulatory T cell immunophysiology, several setbacks have been met in attempting to utilize them for immunotherapy. Drugs such as Rapamycin and CD154 that are designed to halt T cell activation and boost regulatory T cell populations are rendered insufficient in the presence of toll-like receptor (TLR) agonists. TLR agonists therefore result in immunooactivation and transdifferentiation of Tregs into effector T cell phenotypes.

Our understanding of TLR receptors and pathways is substantial, however, relatively little research has been directed towards investigating their effects on regulatory T cell populations. This study’s primary goal was to broaden the current understanding of how TLR activation affects regulatory T cells. In doing so, we aimed to elucidate additional aspects of the TLR mechanisms that result in direct T cell activation and the reduction of Treg numbers. As the investigation moves forward, this will be accomplished by identifying expression of transcription factors and signaling molecules that is initiated upon TLR activation. Our finding that the presence of TLR agonists likely result in the adoption of a Th2 effector phenotype by regulatory T cells is a step towards meeting this goal. It is expected that our in vivo models will elucidate further phenotypic shifts of regulatory T cells associated with the activation of toll-like receptors. It is our hope that a comprehensive understanding of TLR-induced effects on regulatory T cell
expression will result in the development of more effective drug models for inducing
tolerance. Such drugs will likely work by stopping TLR activation from bypassing
therapeutic blockades meant to bolster regulatory T cell populations. Such drug therapy
would represent major progress in transplant research and will likely pave the way for
dramatically increased rates of allograft acceptance.
REFERENCES


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CURRICULUM VITAE

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EDUCATION

Boston University School of Medicine, Boston, MA
Master’s of Science in Medical Science, Expected Graduation May 2015

College of Charleston, Charleston, SC
Bachelor of Science in Biology; Minor in Economics, May 2013
Activities: Biology Club Member
            Treasurer, CofC Club Lacrosse Team
            Organic Chemistry Private Tutor

Southern Methodist University, Dallas, TX
Undeclared Major, 2009 – 2010
Activities: SMU Student Senate

EXPERIENCE

Beth Israel Deaconess Medical Center, Boston, MA
Research Intern- August 2014-Present
- Performed laboratory research to investigate the roles that regulatory T cell plasticity has in the rejection of allograft tissue
- Mouse models were used to study pancreatic islet allograft rejection.
- Used flow cytometric analysis to identify and sort cells of interest
- Worked extensively with other investigators in various projects throughout the lab
- Required the use of oral presentations to explain experimental plans and results

College of Charleston, Charleston, SC
Organic Chemistry Tutor- Spring 2013
- Privately tutored two students in Organic Chemistry 2.
- This involved making lesson plans and meeting with each student once a week for an hour and a half.

**Biosphere Foundation, Bali Indonesia**  
**Research Intern, Summer 2012**

- Joined members of the biosphere foundation to study and collect data on the various ecosystems of Bali, Indonesia.
- Main focus was on the state of coral reefs off the coast of Menjangan. This experience taught me a great deal about the challenge of maintaining a healthy ecosystem while still satisfying the native people’s economic interests.

**University of Miami Bascom Palmer Eye Institute, Miami, FL**  
**Research Intern, May – June 2011**

- Performed research and set up a project pertaining to thermal injuries to the cornea.
- Learned laboratory techniques such as protein extraction, routine protein quantification using spectrophotometry, protein analysis using mass spectrometry, and Western Blot analysis.

**Boca Raton Pediatric Associates, Boca Raton, FL**  
**Clinical Intern, May – July 2010**

- Spent the summer shadowing Dr. Suzanne Laskas, MD.
- Assisted in clinical procedures including physical exams, growth chart analyses, throat test procedure for diagnosis of streptococcal pharyngitis, HCT/HGB finger prick analyses, and sick/well baby exams.

**Premier Eye Care, Delray Beach, FL**  
**Assistant to the Medical Director, May – August 2009**

- Assisted in the development and organization of networks of physicians to work with the company.
- Prepared and presented research aimed at acquiring contracts with major healthcare providers throughout Florida.

**Aker Kasten Eye Center, Boca Raton, FL**  
**Ophthalmic Technician Intern, May – August 2008**

- Shadowed Dr. Kelli Wolper, OD and Dr. Vito Guario, OD during clinical rounds.
- Observed Dr. Alan Aker, MD in surgery.
- Performed common ophthalmic clinical techniques involved in routine eye examinations.
VOLUNTEER ACTIVITIES AND INTERESTS

Aker Kasten Eye Center, Boca Raton, FL
- Wet labs are designed to teach participants phaco-flip cataract surgery.
- Completed approximately 40 surgeries and have familiarity with the procedure and several potential post-op complications.

Caridad Health Center, Boynton Beach, FL
General Assistant, July – August 2010
- The Caridad Center is an organization whose goal is to improve the health, education and living standards of underserved children and families.
- Job entailed bringing patients to examination rooms, filing charts, and helping in any other way possible.
- Learned about the unmet needs of Florida’s underprivileged minority population, as well as the various ongoing efforts to try and improve their current living standards.

College of Charleston, Charleston, SC
Biology Club Member, Spring 2012-Spring 2013