2012

SIGNALING MECHANISMS INVOLVED IN THE GENERATION OF HUMAN PERIPHERAL iTREGS

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Mary Catherine Reneer, Student
Dr. Francesc Marti, Major Professor
Dr. Joe McGillis, Director of Graduate Studies
SIGNALING MECHANISMS INVOLVED IN THE GENERATION OF HUMAN PERIPHERAL iTREGS

________________________________________
DISSERTATION
________________________________________

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Medicine at the University of Kentucky

By
Mary Catherine Reneer
Lexington, Kentucky

Director: Dr. Francesc Martí, Assistant Professor of Microbiology, Immunology and Molecular Genetics
Lexington, Kentucky
2012

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ABSTRACT OF DISSERTATION

SIGNALING MECHANISMS INVOLVED IN THE GENERATION OF HUMAN PERIPHERAL iTREGS

Maintaining balance in the human immune system is critical for the body’s ability to discriminate between foreign and self-antigens. This balance is achieved, in part, by a subpopulation of T cells known as induced regulatory T cells (iTregs). Dysregulation of this population may contribute to the onset and progression of cancer, chronic inflammation and autoimmune diseases. Therefore, manipulation of iTreg development holds promising therapeutic potential; however, studying this vital population has proven difficult due to low numbers, heterogeneous cell populations, substantial phenotypic differences between mouse and human cells, and the high plasticity seen in iTregs. These current limitations have prevented a full understanding of the molecular signaling events that govern their development and function. Our lab has established a novel cell culture system that mimics in vivo human iTreg development. This system allows for the discrimination and comparison of naïve, memory and iTreg T cell populations simultaneously within a single donor. These iTregs exhibit high levels of CD25, FoxP3, CTLA4, GITR, low levels of CD127 and display strong suppressor activity. Using this innovative system, we have demonstrated a rewiring of T cell receptor (TCR) signaling in iTregs compared to conventional T cells. We found that the voltage gated K+ ion channel-Kv1.3 is not active in response to TCR engagement in iTregs, even though Ca^{2+} influx remains intact. Kv1.3 and the linked Src-family kinase Lck were redistributed to the highly active IL2-Receptor (IL2-R) complex. Additionally, we have shown that there is increased AKT protein expression in iTregs versus conventional T cell populations that does not correlate with the TCR-induced increase in its active (phosphorylated) form. This blockage appears to be due to an imbalance of kinase to phosphatase activity in iTregs with a specific TCR-induced inhibition of mTOR activity. We have also demonstrated that AKT accumulation in iTregs leads to its physical association with SMAD3, suggesting a novel, non-enzymatic function of AKT through transcription factor
inhibition. This study sheds light on the reciprocal cross talk between the IL-2R and TCR signaling pathways and uncovers the mechanism of AKT blockade in primary human iTregs, thus opening novel avenues for therapeutic manipulation.

Keywords: regulatory T cells, TCR signaling, AKT, SMAD3, Kv1.3

Mary Catherine Reneer

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24 September 2012

Date
SIGNALING MECHANISMS INVOLVED IN THE GENERATION OF HUMAN PERIPHERAL ITREGS

By

Mary Catherine Reneer

Francesc Marti, Ph.D.
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Joe McGillis, Ph.D.
Director of Graduate Studies

24 September 2012
Date
I dedicate this dissertation to my parents, sister, and husband who have been my support and driving force through this entire process.
ACKNOWLEDGMENTS

Foremost, I would like to express my gratitude to my Ph.D. mentor Dr. Francesc Martí. His guidance and constructive assistance have provided the basis for my thesis project. He helped me to learn, understand and perform science with an inquisitive and critical mind. He has been an integral part in my becoming the scientist I am today. I wish to express my sincere thanks to my committee members Drs. Don Cohen, Subbarao Bondada, Leslie Crofford and John D’Orazio for their help in providing insights that guided and challenged my thinking, substantially improving my scientific thinking and thesis work. My special thanks to Dr. Alan Kaplan and Dr. Beth Garvy for their guidance as chairs of the department during my graduate work. Thank you to Dr. Sergey Artiushin for agreeing to be my external examiner.

I would like to additionally thank the fellow lab members: Mr. Gavin Ellis, Ms. Alejandra Catalina Vélez-Ortega, and Ms. Andrea McCool for all their help during my last four years in the lab. A special thank you to Gavin Ellis as words cannot express how much I have learned from him and how much he has impacted my time in the lab. Without Gavin, lab would have been in no way been as fun or exciting. A special thank you to Alejandra Catalina Vélez-Ortega from whom I have learned so much about flow cytometry and who will be one of my best friends for life. Last but not least I would like to thank Andrea McCool for all of her help during my graduate work without whom my work in the lab would have been twice as hard. I really appreciate all of the past and
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<tbody>
<tr>
<td>Treg</td>
<td>Regulatory T cells</td>
</tr>
<tr>
<td>iTreg</td>
<td>Induced Regulatory T cells</td>
</tr>
<tr>
<td>nTreg</td>
<td>Natural Regulatory T cells</td>
</tr>
<tr>
<td>TCR</td>
<td>T Cell Receptor</td>
</tr>
<tr>
<td>IL-2R</td>
<td>IL-2 Receptor</td>
</tr>
<tr>
<td>PAMPS</td>
<td>Pathogen-Associated Molecular Patterns</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of Differentiation</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen Presenting Cell</td>
</tr>
<tr>
<td>T\textsubscript{H} cells</td>
<td>Helper T Cells</td>
</tr>
<tr>
<td>IFN-\gamma</td>
<td>Interferon Gamma</td>
</tr>
<tr>
<td>TNF-\beta</td>
<td>Tumor Necrosis Factor Beta</td>
</tr>
<tr>
<td>IL-</td>
<td>Interleukin</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic Cell</td>
</tr>
<tr>
<td>CD25</td>
<td>IL-2 Receptor alpha chain</td>
</tr>
<tr>
<td>FoxP3</td>
<td>Forkhead Winged Helix Transcription Factor</td>
</tr>
<tr>
<td>IPEX</td>
<td>Immunodysregulation Polyendocrinopathy and Enteropathy</td>
</tr>
<tr>
<td>CTLA4</td>
<td>Cytotoxic T Lymphocyte Antigen 4</td>
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<tr>
<td>GITR</td>
<td>Glucocorticoid-Induced Tumor Necrosis Factor Receptor</td>
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<td>CD127</td>
<td>IL-7 Receptor Alpha Chain</td>
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<td>TGF-\beta</td>
<td>Transforming Growth Factor Beta</td>
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<tr>
<td>MS</td>
<td>Multiple Sclerosis</td>
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<td>IBD</td>
<td>Inflammatory Bowel Disease</td>
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<tr>
<td>SLE</td>
<td>Systemic Lupus Erythomatosus</td>
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<tr>
<td>RA</td>
<td>Rheumatoid Arthritis</td>
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<tr>
<td>Zap70</td>
<td>Zeta-Chain-Associated Protein Kinase 70</td>
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<tr>
<td>LAT</td>
<td>Linker of Activated T Cells</td>
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<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-Kinase</td>
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<td>IP\textsubscript{3}</td>
<td>Inositol 1,4,5-triphosphate</td>
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<td>NF-kB</td>
<td>Nuclear Factor kappa-light-chain-enhancer of Activated B cells</td>
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<td>AP-1</td>
<td>Activator Protein 1</td>
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<td>NFAT</td>
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<td>PKC</td>
<td>Protein Kinase C</td>
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<td>PKA</td>
<td>Protein Kinase A</td>
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<td>PKB</td>
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<td>AGC</td>
<td>A/Protein Kinase G/Protein Kinase C</td>
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<tr>
<td>PH</td>
<td>Pleckstrin Homology Domain</td>
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<td>HM</td>
<td>Hydrophobic Motif</td>
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<td>PtdIns (3,4,5)P\textsubscript{3}</td>
<td>Phosphatidylinositol-3,4,5-triphosphate</td>
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<td>PtdIns-4P</td>
<td>Phosphatidylinositol-4-monophosphate</td>
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<td>T308</td>
<td>Threonine 308</td>
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<td>Serine 473</td>
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<td>PDK1</td>
<td>Phosphoinositide-Dependent Kinase-1</td>
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<td>mTOR</td>
<td>Mammalian Target of Rapamycin</td>
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<td>mTORC1</td>
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<td>mTORC2</td>
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<td>RTK</td>
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<td>Protein Phosphatase 2</td>
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<tr>
<td>ETPs</td>
<td>Early Thymic Progenitors</td>
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<td>DN</td>
<td>Double Negative</td>
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<td>FoxO</td>
<td>Forkhead Box Protein</td>
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<td>Cyclic Adenosine 3’5’-monophosphate-responsive element binding protein</td>
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<td>SBE</td>
<td>SMAD-Binding element</td>
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<td>PBMC</td>
<td>Peripheral Blood Mononuclear Cells</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
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</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
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<td>2-ME</td>
<td>β-mercaptoethanol</td>
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<td>CFSE</td>
<td>Carboxyfluorescein Succinimidyl Ester</td>
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<td>CCR2</td>
<td>Chemokine (c-c motif) Receptor 2</td>
</tr>
<tr>
<td>LFA-3</td>
<td>Lymphocyte Function-Associated Antigen 3</td>
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<tr>
<td>PLC-γ</td>
<td>Phospholipase C Gamma 1</td>
</tr>
<tr>
<td>Gal3</td>
<td>Galectin-3</td>
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<tr>
<td>Grb-2</td>
<td>Growth Factor Receptor-Bound Protein 2</td>
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</tbody>
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Chapter 1: INTRODUCTION

All higher organisms are challenged by pathogens in their surrounding environment continuously attempting to invade the body ultimately resulting in an imbalance of immune homeostasis. Adding to the complexity of the interaction between the host and invasive organisms, higher organisms naturally support commensal bacteria at mucosal surfaces and skin through a highly evolved and specific symbiotic relationship. This commensal relationship requires the ability to distinguish between desirable and undesirable microorganisms in order to favor the survival of beneficial symbionts and simultaneously eradicate pathogenic organisms. The human immune system consists of a variety of cell populations each contributing to the tolerance of self and symbiotic cells and to the defense against foreign pathogens. It can generally be divided into two distinct, yet complementary subdivisions: innate immunity and adaptive immunity. The innate division of the immune system generates the first line of defense against an infection or insult by reacting to pathogens in a non-specific but rapid manner. Cells of the innate immune system typically respond to pathogen-associated molecular patterns (PAMPs) expressed by infectious agents that are conserved across groups of microscopic organisms and are not generally specific to species or strain. Conversely, the adaptive response requires an antigen-specific recognition of pathogens that can take several weeks to develop. Though the generation of a primary adaptive immune response is slow, it generates a long-term protective memory that enables a rapid response upon additional encounters with a pathogen bearing an identical antigen. The adaptive immune response can be further subdivided into two types: humoral, which is mediated by B cell-
produced antibodies; and cell-mediated, which involves the generation and activation of antigen specific T cells. Combined with the innate immune response, both humoral and cell-mediated adaptive responses are essential for the immune system as a whole to fight off invading pathogens.

1.1 T lymphocytes – Effector T cells

The vital function of T cells in the defense against foreign pathogens is most potently demonstrated by a loss or defect in T cell populations which leaves individuals more vulnerable to infections. Two major subsets of peripheral mature T cells can be identified by their mutually exclusive expression of cluster of differentiation (CD)8 and CD4 cell surface molecules (1). Most CD8+ T cells display cytotoxic effector function once activated in which they induce cell death in host cells infected with a pathogenic microorganism. CD8+ T cells recognize and lyse cells expressing the pathogenic antigen associated with major histocompatibility complex (MHC) class I molecules. On the other hand, CD4+ T cells recognize foreign antigens in the context of class II self MHC, which enables them to respond primarily to professional antigen presenting cells (APC). Once activated, most effector CD4+ T cells become helper T cells (T_{H} cells) that provide essential additional signals to activate and direct other cells to respond aggressively to an infectious agent (2). Historically, CD4+ T_{H} cells were classified as T_{H}1 or T_{H}2, however recently a switch to characterization of T_{H} cells based on their cytokine expression signature has gained popularity. T_{H}1 cells participate in cell-mediated immunity and are critical for the control of intracellular pathogens such as viruses and certain intracellular
bacteria through the production and secretion of interferon (IFN)-γ and tumor necrosis factor beta (TNF-β) (3). T_{H}2 cells direct B cell activation and antibody production as well as basophilic and eosinophilic inflammation through the secretion of Interleukin (IL)-4, IL-13 and IL-5 (3). T_{H}17 cells function to protect skin surface and intestine against extracellular bacteria through the production and secretion of IL-17 (4). All T_{H} cell subsets listed above play a critical role in specific recognition of and in organizing a response against foreign pathogens. Though classic definitions of T cell lineage commitment are still in widespread use, the status quo has recently been challenged by evidence highlighting functional and developmental plasticity of T cells. These recent studies clearly demonstrate that T cells exhibiting an individual T_{H} subset phenotype are able to transition between T_{H} lineages when needed and when in the presence of a cytokine milieu permissive to the transition (5). Effector T cells form an integral part of the immune response that maintains flexibility to adapt to the ever-changing immune homeostatic balance.

1.2 Regulatory T cell subsets

Overactivation of effector T cells in the immune system can lead to an excessive, pathological inflammatory response and/or to autoimmune disease (6). Maintaining a balance between effective immune response and peripheral tolerance is achieved, in part, by regulatory T cells (Tregs) (7, 8) through direct cell to cell contact, soluble factors, metabolic disruption or indirect effect mediated by dendritic cell (DC) suppression (9-11). First known as “suppressor” cells, for over a decade it remained unclear the true identity of these anti-inflammatory cells due to an inability to distinguish them from other
conventional T cells. A turning point of Treg research came in 1995 when the link between suppressor function and cells that expressed high levels of the IL-2 receptor alpha chain (CD25) was made (12). Additionally, in 2003 the identification of the forkhead winged helix transcription factor (FoxP3) as a critical regulator of Treg development and function helped to establish a Treg phenotype (13-16). Deficiencies in FoxP3 underlie the lymphoproliferation and multiorgan autoimmunity of scurfy mutant mice and human patients with Immunodysregulation Polyendocrinopathy and Enteropathy, X-linked syndrome (IPEX) (17, 18). IPEX usually affects males causing them to rarely live beyond infancy and to suffer from severe enlargement of the secondary lymphoid organs, insulin-dependent diabetes, eczema, food allergies and concomitant infections (19). Despite their critical involvement in immune homeostasis, Tregs constitute only about 1-5% of the circulating T cells in the human body. Two main subsets have been distinguished according to their origin: naturally occurring, thymically selected natural Tregs (nTregs), and peripherally induced Tregs (iTregs) differentiated from circulating conventional CD4+ T cells. These two subsets are phenotypically indistinguishable in their high expression of CD25, cytotoxic T lymphocyte antigen 4 (CTLA4), glucocorticoid-induced tumor necrosis factor receptor (GITR) and FoxP3, and lack of the alpha chain of the IL-7 receptor (CD127). Unfortunately, despite intensive research, there are still no definitive surface or intracellular markers that clearly distinguish Tregs from conventional T cells in humans, although nTregs differ from iTregs in a variety of ways (Table 1). While high affinity interactions with cognate self-peptide-MHC complexes and strong CD28 co-stimulation drive the development of
nTregs, iTregs require weaker, suboptimal T cell receptor (TCR) stimulation and can be generated in the absence of CD28 co-stimulation (20). In addition, differentiation of iTregs, but not nTregs, requires IL-2 and transforming growth factor-β (TGF-β) (21). nTregs develop a TCR specificity for self-antigens in the thymus whereas the iTreg repertoire is more specific for tissue and foreign antigens (20). This dichotomy may account also for their different regulatory activities, with nTregs preferentially involved in preserving self-tolerance and iTregs being more functionally active at inflammatory sites.
Table 1. Comparison of nTregs and iTregs.

<table>
<thead>
<tr>
<th>Feature</th>
<th>Natural Tregs</th>
<th>Induced Tregs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Site of Induction</td>
<td>Thymus</td>
<td>Periphery</td>
</tr>
<tr>
<td>Generation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-2 and TGF-β required</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>CD28 Dependent Development</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>TCR Specificity</td>
<td>Self-antigens in thymus</td>
<td>Tissue-specific and foreign antigens</td>
</tr>
<tr>
<td>Maintenance</td>
<td>IL-2 and TGF-β</td>
<td>IL-2 and TGF-β</td>
</tr>
</tbody>
</table>

Modified with permission from (20, 21).
Careful attention must be paid to the differences between mouse and human Tregs. While FoxP3 is considered the definitive marker for mouse Tregs, the expression of FoxP3 in human is not confined to Tregs, but can be also induced in activated, conventional T cells (22). This observation added further confusion as to whether circulatory FoxP3+ Tregs are thymic in origin and persist in the periphery, or whether they are induced during antigen-specific stimulation (23).

Key evidence for the relevance of peripheral generation of human Tregs came from the work of Vukmanovic-Stejic et al. The authors proposed that antigenic persistence induces a continuous generation of short-lived and prone-to-apoptosis Tregs from a population of rapidly dividing, highly differentiated memory CD4+ T cells (24, 25). Since the number and function of CD4+CD25+FoxP3+ Tregs is maintained even during senescence in humans (23), thymic nTregs cannot account for the lifelong existence of human Tregs supporting the hypothesis that Tregs in adult humans develop from a pool of circulating peripheral T cells. Recent evidence emphasizes plasticity of T H cells, with this in context, iTregs can be generated from T H1, T H2, and T H17 effector cells as well as from naïve cells (26) (Figure 1.1). In fact, CD103+ DCs from the mesenteric lymph nodes can induce the differentiation of naïve CD4+ T cells into iTregs in the mucosal environment of the gut, providing a mechanism by which functionally specialized gut-associated lymphoid tissue DCs can extend the repertoire of Treg cells (27). These findings are consistent with iTreg differentiation being tailored according to specific microenvironment requirements, thus ensuring the physiological ability to control immune homeostasis when and where needed. In addition to their critical function
in maintaining immune homeostasis after infection or inflammatory response, iTregs are also considered major contributors to the failure of immunosurveillance during cancer progression as the tumor microenvironment is especially favorable for the generation of iTregs (28).
Figure 1.1: Origin and maintenance of peripheral CD4+CD25- Treg cells.
Naturally occurring thymic-derived regulatory T cells (nTregs) develop in thymus as a separate lineage but display limited capacity for self-regeneration. Therefore, peripheral Treg homeostasis is likely to be preserved by the continuous production of Tregs from another population. Experimental evidence supports the prevalence of the newly generated iTreg in the maintenance and control of peripheral Treg homeostasis (23).
This high degree of developmental plasticity in iTregs also makes them functionally unstable. Xu et al. (29) and Yang et al. (30) each found independently that a fraction of peripheral FoxP3+ T cells lose FoxP3 expression and produce IL-17 upon *in vitro* stimulation in the presence of IL-6. Also, when highly purified FoxP3+ cells were transferred into RAG-/- mice, approximately half of the cells lost their FoxP3 expression and produced IFN-γ, IL-2 and IL-17 (31, 32). In addition, FoxP3+ T cells may acquire TH cell features without losing FoxP3 expression. These hybrid FoxP3+RORγt+IL-17+ or FoxP3+T-bet+IFN-γ+ cells have been characterized as being in a transitional state between Treg and TH phenotypes (33). The issue of Treg plasticity is controversial and it remains to be established whether it reflects true lineage reprogramming of committed Tregs or is a dynamic transition of an uncommitted population of FoxP3+ T cells (34). This high degree of plasticity in the iTreg developmental program represents an additional challenge to the inherent difficulties associated with the study of human Tregs, such as small numbers of cells, impure populations and phenotypic differences between animal and human models, which is particularly important when considering Tregs in therapeutic treatment for human disease. These intrinsic limitations have prevented a comprehensive understanding of the differential signaling events that govern the development and function of Tregs.

### 1.3 Link between dysregulation of Tregs and human disease

Dysregulation of the Treg population has been implicated in autoimmune and chronic inflammatory diseases, infections and cancer (35). Therefore understanding the
link between the role of Tregs and the disease onset will be critical for treatment. In multiple sclerosis (MS), controversial results have been published on the role of CD25⁺FoxP3⁺ Tregs. The level of Tregs in the blood of MS patients and healthy controls were compared and found to be either normal or decreased in MS patients (36-39), however others have postulated that even if the frequency of Tregs is normal, the cells may not be functioning normally and are unable to control the activation and proliferation of the pathogenic T cells (40). Recently, Dalla Libera et al. found that Tregs were significantly decreased in stable MS patients but were restored to normal levels during an acute attack, suggesting that the role of Tregs is to react to the inflammation during a relapse and not necessarily the cause of the relapse (39). It is clear that Tregs do contribute to the disease in humans, but the extent of their involvement needs to be further investigated. Inflammatory bowel diseases (IBD) like ulcerative colitis and Crohn’s disease have also been linked to dysregulation of Treg populations (41). IBD is a family of chronic diseases characterized by infiltration of inflammatory cells into the lamina propria of the intestinal tract. Patients with IBD appear to have relatively reduced numbers of Tregs in the blood and in the colon; however these Tregs still appear to be functional in vitro (41). Additionally, it has been found that in the experimental model of diabetes using NOD mice, treatment with islet-specific Tregs can block diabetes months after initiation, suggesting that the endogenous Treg cells could be dysfunctional leading to the onset of the disease (42). Systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) both arise due to a failure of immunological self-tolerance and have both been link to deficiencies in the Treg population (43). In RA it is has been found that there
are relatively normal levels of Tregs in the patients, but that the pathogenic T cells in the microenvironment do not appear to be responsive to Treg suppression (44). Lastly, in graft versus host disease, treatment with Tregs has also been found to be beneficial in limiting the disease lethality (45). These studies implicated that regulatory T cell development, control and function may offer insight into the onset and treatment of autoimmune and chronic inflammatory diseases, asthma and cancer.

1.4 T cell receptor signaling

To sense the changes in their surrounding microenvironment, T cells utilize a variety of specialized cell membrane receptors that interact with ligands in the local milieu. The ligand-receptor interaction initiates a chain of intracellular biochemical reactions –pathways that relay, amplify and distribute those initial signal inputs into the cell. Cross talk among multiple, simultaneously active pathways, together with positive and negative feedback activation loops, produce a complex signaling network that is tightly coordinated and integrated to summon the adequate cell response. This response is elaborated in the cell nucleus and mediated by the action of transcription factors through the regulation of gene expression and protein synthesis (6). T cell activation begins with the specific recognition of antigen displayed on MHC heterodimers on the surface of antigen presenting cells by the TCR (Figure 1.2) (46). The TCR is anchored in the cell membrane and consists of two chains alpha (α) and beta (β). TCR associates into a complex with other molecules like CD3, which possess distinct chains (γ, ζ, δ and ε). Engagement of the TCR, as well as other co-stimulatory receptors on the T cell like CD-
28, with the APC triggers a rearrangement of the T cell membrane and the formation of the immunological synapse (47). The binding of the TCR to MHC/antigen complex to the TCR complex leads to a conformational change in CD3ζ, allowing the protein tyrosine kinase Lck to phosphorylate the immunoreceptor tyrosine-based activation motifs (ITAMs) on CD3, subsequently recruiting Zeta-chain-associated protein kinase 70 (Zap70). Zap70 will then be phosphorylated and activate the scaffolding protein linker of activated T cells (LAT) which will further recruit other adaptor proteins eventually leading to the activation of phosphatidylinositol 3-kinase (PI3K), and generation of inositol 1,4,5-trisphosphate (IP₃), among other second messengers. IP3 will then diffuse through the cytosol and induce Ca²⁺ to be released from the endoplasmic reticulum. This complex series of events will prompt activation of several downstream transcription factors such as: activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), activator protein 1 (AP-1), nuclear factor of activated T-cells (NFAT), along with others leading to T cell differentiation, T cell proliferation and production of inflammatory cytokines generating a effector T cell response (48).
Figure 1.2: TCR Signaling.
Schematic outline of TCR Signaling as discussed in the text. Reproduced with permission from (46).
1.5 Protein kinase AKT

The serine/threonine-specific protein kinase AKT, also known as protein kinase B (PKB) is gaining recognition as the site of a major crossroad in numerous cellular signaling pathways involved in the regulation of vital cell functions such as differentiation, proliferation, glucose metabolism and survival. As such, dysfunctional AKT is observed in many pathological conditions, including cancer (49), autoimmune disease (50), neurodegenerative conditions (51) and muscle atrophy (52), making it an attractive pharmacological target in current drug discovery programs.

AKT research can be traced back to 1977 when Staal et al. (53) reported the isolation and characterization of AKT8, a murine virus that caused a high incidence of spontaneous lymphoma. The viral oncogene was termed v-AKT and, later the cellular homolog was identified as c-AKT in 1991 (54). Additionally, two other groups looking for members of the protein kinase C (PKC) and protein kinase A (PKA) superfamilies, identified a gene that encodes a serine/threonine protein kinase which they named PKB (55, 56). c-AKT and PKB were later identified as the same protein. Further studies demonstrated the critical involvement of AKT in growth factor signaling and oncogenesis. In 1995, it was reported that the lipid kinase phosphatidylinositol 3-kinase (PI3K) is directly upstream of AKT (57, 58) thus establishing a linkage of the PI3K-AKT pathway to metabolic functions such as protein and lipid synthesis, carbohydrate metabolism and transcription. As the field evolved, a new perspective on the role of AKT in growth, survival and metabolism became established and, with it, the foundation for the current compelling interest in the molecular regulation of AKT activity.
1.5.1 Protein structure of AKT

AKT belongs to the cAMP-dependent protein kinase A/protein kinase G/protein kinase C (AGC) superfamily of protein kinases. Each AGC member exhibits similar enzymatic function and structural homology within their catalytic domains (59). Three different AKT genes have been identified in mammalian cells: AKT1/PKBα, AKT2/PKBβ and AKT3/PKBγ (55, 60-64). Although AKTs are expressed in all eukaryotes, the specific isoforms and level of expression varies depending on the tissue type. AKT1 is the dominant isoform in the majority of tissues and is critical during embryonic development (65-67). AKT2 is expressed mostly in insulin-responsive tissues, has non-redundant functions in glucose homeostasis (60), and AKT3 is restricted to testis and brain and is required for postnatal brain development (62-64).

The three isoforms of AKT share a high degree of amino acid identity (~85%) and a common structural pattern that includes an amino terminal pleckstrin homology (PH) domain, a central kinase domain and a carboxyl-terminal regulatory domain with a hydrophobic motif (HM) (Figure 1.3). The PH domain is a protein module of approximately 120 amino acids best known for its ability to bind to cell membrane phosphoinositides. The AKT PH domain preferentially recognizes phosphatidylinositol-3,4,5-triphosphate (PtdIns (3,4,5)P_3) and, with three fold lower affinity, PtdIns (3,4)P_2 (68, 69). Unlike PtdIns (4,5)P_2 and PtdIns-4P, which are constitutively produced in cells, PtdIns (3,4,5)P_3 and PtdIns (3,4)P_2 are detected only in stimulated cells (70) thus providing an additional mechanism to regulate the recruitment of proteins such as AKT to the membrane. Next to the PH domain, the kinase domain shares a high similarity with
other AGC kinases and contains the catalytic function of AKT that exhibits a preference to phosphorylate the canonical consensus sequence RXRXX(S/T) \(^{(71)}\). It contains the regulatory threonine 308 (T308) of which phosphorylation is required for AKT activation. AKT possesses a carboxyl-terminal regulatory domain characteristic of the AGC kinase family with an FXX(F/Y)(S/T)(Y/F) hydrophobic motif. This sequence contains serine 473 (S473), the second regulatory residue of which phosphorylation is necessary for full activation of the kinase. Together these regions make up a protein of approximately 56 kDa \(^{(72)}\).
Figure 1.3

Figure 1.3: Structure of AKT
(A). Depicted is the structure of AKT1. AKT consists of three domains: the PH domain, the kinase domain and the COOH-terminal regulatory domain with a hydrophobic motif (HM). The kinase domain contains T308, one of two residues that need to be phosphorylated in order for AKT to become active. The second residue, S473, is located in the hydrophobic motif of AKT. Reproduced with permission from (6).
1.5.2 Activation of AKT

Activation of AKT is a multi-step process that requires the receptor-ligand dependent activation of PI3K. PI3K is the lipid regulator that phosphorylates PtdIns (4,5)P\(_2\) and, with lower efficiency, PtdIns 4P, at the 3’-OH position of the inositol ring to produce PtdIns (3,4,5)P\(_3\) and PtdIns (3,4)P\(_2\), respectively (Figure 1.4). In the initial step of its sequential activation, AKT translocates to the cell membrane through the PH-mediated binding to PtdIns (3,4,5)P\(_3\). This binding generates a conformational change in AKT that exposes the two regulatory phosphorylation sites, T308 and S473. The fact that PH domains of Phosphoinositide-Dependent Kinase-1 (PDK1) and AKT share their high affinity for PtdIns (3,4,5)P\(_3\) favors their physical close proximity and enhances the efficiency of the PDK1-mediated phosphorylation of T308 in AKT. Phosphorylation of T308 only partially activates AKT and it is not yet clear whether it is a necessary prior event for phosphorylation of S473 (73). In order to reach full activation, AKT must be phosphorylated at both T308 and S473. The identity of the kinase responsible for phosphorylation of S473 remains controversial and several candidates have been suggested, including PDK1/2 and the mammalian target of rapamycin (mTOR) associated within the mTOR complex-2 (mTORC2) (74-78). mTOR, the kinase that is upstream of AKT as mTORC2, assembles within mTORC1, which is a direct, downstream AKT substrate. AKT-induced phosphorylation of other members in both mTORC1/2 complexes results in opposite effects on the AKT pathway. While phospho-TSC1-2 sustains a positive feedback loop between mTORC2 and AKT (79), in some instances (i.e., the insulin receptor pathway) AKT phosphorylation of mTORC1/2
mediates the inhibitory feedback loop within the PI3K-AKT-mTORC1 axis (80). This complex interplay between mTOR and AKT, mediated by mTORC1 and mTORC2 coordinates and diverts the AKT signaling to specific sets of downstream pathways that will define the functional response of the cell.
Figure 1.4: Regulation of AKT Activation

Upon ligand binding to receptor tyrosine kinase (RTK), PI3K is recruited to the membrane where it phosphorylates its substrate PtdIns (4,5)P₂ leading to the formation of PtdIns (3,4,5)P₃. PtdIns (3,4,5)P₃ induces AKT translocation to the membrane by binding to its PH domain and causing a conformational change that allows T308 and possibly S473 to become accessible to PDK1 and mTOR for phosphorylation. Once phosphorylated, full activation of AKT results in the regulation of multiple cellular events related to cell proliferation, growth and survival. AKT activity is negatively regulated by the action of PtdIns phosphatases PTEN and SHIP whose activity reduces the availability of the AKT-activating PtdIns (3,4,5)P₃. Lastly, PP2A and PHLPP directly target AKT by dephosphorylating phospho-T308 and phospho-S473, respectively, thus reverting AKT to an inactive conformation. Reproduced with permission from (6).
Overactivation of AKT can lead to the loss of proliferative and cell survival control that occurs in a wide array of solid tumors and hematopoietic malignancies. To maintain proper physiological function, AKT activity needs to be exquisitely balanced. Cells counterregulate AKT function via distinct mechanisms, including the degradation of PtdIns (3,4,5)P$_3$ by inositol phosphatases. Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) antagonizes PI3K activity and dephosphorylates the 3’-OH, generating PtdIns (4,5)P$_2$ (81). The SH2-containing inositol polyphosphate 5-phosphatase 1 (SHIP1) exhibits 5’-OH phosphatase activity to produce PtdIns (4,5)P$_2$ (82, 83). De-phosphorylation of PtdIns (3,4,5)P$_3$ by PTEN and SHIP1 leads to the formation of inositol lipids whose binding affinity for AKT is significantly lowered. The PH domain-only protein PH-like domain family A member 3 (PHLDA3) competes with AKT for binding to both PtdIns (3,4,5)P$_3$, and PtdIns (3,4)P$_2$, and the overexpression of PHLDA3 may block the localization of AKT to the membrane (84). In addition, AKT can be directly targeted by the action of phosphatases such as protein phosphatase 2 (PP2A) and the PH domain leucine-rich repeat protein phosphatase (PHLPP). While PP2A preferentially dephosphorylates AKT at T308 and, under certain conditions, also targets phospho-S473 (85-89), PHLPP dephosphorylates AKT specifically at S473 (Figure 1.4) (90-92).

1.5.3 Pharmacological targeting of the AKT pathway

The physiological relevance of AKT and the potential involvement of the AKT pathway in numerous pathological conditions have attracted major interest in AKT as a
pharmacological target. One such drug is the fungal metabolite rapamycin, which is currently approved for use in humans as an immunosuppressant to prevent rejection following organ transplantation. Rapamycin and its analogs (rapalogs), affect the AKT pathway by directly binding to the FKBP12 binding protein, which interacts preferentially with mTORC1 and blocks its downstream signaling activity. In some instances, inhibiting mTORC1 may result in pernicious effects by breaking the S6K-dependent inhibitory feedback that restrains the PI3K-induced AKT activation (i.e. eliciting the opposite intended outcome) (93). Consequently, alternative therapeutic strategies to rapamycin have been proposed by combining the inhibition of mTOR function and AKT activation. In this context, the strong structural similarities between the catalytic domain of mTOR and the p110 subunit of PI3K (94) facilitate the dual inhibitory activity of new chemical compounds, distinct from rapalogs, that are currently being tested in clinical trials (clinicaltrial.gov ID number NCT00620594). The most common PI3K-specific inhibitors, the synthetic compound Ly294002 (95) and the fungal metabolite wortmannin (96), have been standard reagents in the experimental characterization of PI3K signaling, but their therapeutic use has been disregarded (97) and the attention has been shifted to a new generation of PI3K inhibitors that target the different isoforms of the regulatory p110 subunit. The potential therapeutic benefit of PtdIns analogs has also been debated. Nevertheless, despite their capacity to disrupt AKT activation, they may also generate specificity problems with respect to other PH domain-containing proteins. In contrast, the direct inhibition of AKT is considered a very attractive but challenging therapeutic option, either alone or in combination with other
forms of therapy. Recently, several ATP-competitive inhibitors and novel allosteric inhibitors (PH domain-dependent small molecules) with selectivity for individual AKT isoforms are being actively characterized, some of which are in early phase of clinical trials or in clinical development (98-102).

Active AKT profoundly affects cellular processes by phosphorylating numerous substrates. Consensus motif analyses reveal thousands of potential substrates for the enzymatic activity of AKT which can cause positive or negative regulation of the substrate. However, the consequences of phosphorylation by AKT on substrate function, localization or protein stability has only been characterized for approximately 50 substrates to-date (103).

1.6 The role of AKT in conventional T lymphocytes

1.6.1 AKT in T cell development

The thymic microenvironment directs the development, education and selection of T cells through the sequential integration of multiple extracellular cues that will ultimately generate the mature functional pool of circulating T cells. The first step in T cell development is the migration of early thymic progenitors (ETPs) from bone marrow to the thymus (104). Once in the thymus, ETPs transition through four stages as double negative (DN) T cells (DN1-4) before becoming double positive (DP) CD4⁺CD8⁺ T cells (105). The emergence of a mature T cell requires that the developing thymocytes pass through several pre-T cell receptor (pre-TCR)/TCR dependent selection events, the first of which takes place at the DN3 stage, followed by positive and negative selection during
the DP stage (106). Only DN3 thymocytes that express the newly created pre-TCRβ chain will survive – a process known as β-selection (107, 108). During the subsequent DP stage, additional positive and negative selection occurs in which phenotypic allelic exclusion at the TCRα locus is regulated and selectivity against T cells that are specific for self-peptide/MHC-complexes occurs (106, 109, 110). Although widely studied, the complete picture of signaling events and molecular mechanisms that control thymocyte differentiation is not well characterized. Experimental evidence suggests that AKT is essential during the transition from DN to DP, β-selection, allelic exclusion, and the subsequent proliferation that follows DP transition, but the identification of specific pathways in which AKT is involved is still the focus of very active research (111-116). A better understanding of the molecular events that control thymocyte development and T cell maturation will likely shed light upon the etiology and onset of a plethora of T cell-related pathologies, including autoimmune diseases and malignant transformations.

1.6.2 AKT in peripheral CD4⁺ effector T cell differentiation

AKT signaling is involved in the peripheral differentiation of distinct effector Th cell subsets. Arimura et al. (117) found that the expression of a constitutively active form of AKT induced Th1 differentiation in C57BL/6 mice; however, AKT promoted Th2 differentiation in BALB/C mice. Conversely, Kane et al. (118) reported that CD28-induced AKT up-regulated the Th1 cytokines IL-2 and IFN-γ but not Th2 cytokines in BALB/C mice. These discrepancies were reconciled by the findings of Lee et al. (119) who investigated mTORC2. These authors created mice with a conditional deletion of rictor on C57BL/6 background, an essential subunit in the mTORC2 complex, and
reported that both T\textsubscript{H}1 and T\textsubscript{H}2, but not T\textsubscript{H}17 differentiation, were impaired. Additionally, they found that complementation with constitutively active AKT rescued only T\textsubscript{H}1 differentiation in mTOR deficient mice, whereas activated PKC-\(\theta\) restored T\textsubscript{H}2 cells, implying that mTOR-dependent AKT plays a pivotal role in the development of the T\textsubscript{H}1 subset, but not T\textsubscript{H}2. AKT has been also reported to be crucial for the expression of T\textsubscript{H}17 cytokines in CCR6\(^+\) human memory T cells (120).

1.6.3 AKT activation in conventional T cells

The plethora of stimuli and pathways that regulate AKT function underlines its crucial role in T cells. One of the first events was documented by Reif et al. (121), who showed that the addition of IL-2 to a human T cell line lead to stimulation of AKT in a PI3K-dependent fashion. Later, Lafont et al. (122) reported that TCR ligation, like IL-2 ligation, also promoted a sustained, PI3K-dependent activation of AKT that lasts several hours (122). Co-stimulation through the CD28 receptor is required for optimal TCR-dependent naïve T cell activation, cytokine production, cell survival and proliferation (123). Parry et al. (124) reported that upon CD28 co-stimulation, TCR-induced PI3K-dependent AKT activation increased substantially in primary human T cells. Upon activation, AKT integrates and relays multiple pathways that dictate downstream events responsible for defining the outcome of the T cell function and fate. Phosphorylation of downstream substrates of AKT leads to activation or deactivation of a myriad of regulatory events in T cells. Among them, glycogen synthase kinase 3 (GSK-3) modulates the synthesis of glycogen in lymphocytes. GSK-3 is constitutively activated in the absence of growth factors and directly regulates nuclear factor of activated T-cells.
NFAT, a family of transcription factors involved in cytokine gene induction (125). NFAT activity is controlled by a nuclear import/export cycle governed by phosphorylation/de-phosphorylation (126). NFAT is directly phosphorylated by GSK-3, and the phosphorylation and inactivation of GSK-3 by AKT indirectly promotes NFAT-dependent synthesis of pro-inflammatory cytokines in T cells (Figure 1.5). Similar to NFAT, the eukaryotic initiation factor 2 (eIF-2B) involved in the translational control of protein synthesis, is another substrate of GSK-3 that is rescued by AKT (127).
Figure 1.5: Metabolic functions of AKT in conventional T cells
Schematic outline of the downstream protein targets of AKT in conventional T cells as discussed in the text. Reproduced with permission from (6).
AKT is also functionally linked with the transcription factor NF-κB (Figure 1.5), which is involved in T cell development, maturation and proliferation (128). Kane et al. (129) found that catalytically active AKT is necessary for the degradation of the NF-κB inhibitor IκB, although it is still controversial whether or not AKT alone is sufficient to induce NF-κB (130, 131) and whether or not IκB is a direct target of AKT (132, 133). Regardless of the precise mechanism, it is clear that the PI3K/AKT/NF-κB signaling axis is operative in activated T cells.

The FoxO forkhead family of transcription factors plays an important role in cell proliferation and differentiation and is another major target of AKT kinase (134). FoxO3 is involved in cell cycle regulation and survival of T cells by inducing the IL-2-dependent expression of the cell cycle inhibitor p27kip1 and the pro-apoptotic Bcl-2 family member Bim (135) (Figure 1.5). Like other transcription factors, FoxO activity is regulated by its nuclear localization, and its phosphorylation by AKT precludes its nuclear accumulation and activity (134, 136). In addition, AKT has been found to modulate the activity of another member of the Bcl-2 family, the pro-apoptotic BAD (137, 138). Upon phosphorylation, BAD dissociates from Bcl-2 and forms a very stable complex with 14-3-3 protein, which allows the free Bcl-2 to associate with and inhibit the pro-apoptotic effect of Bax. AKT has been found to regulate cell survival through other pathways, including the NF-κB-dependent upregulation of Bcl-XL (139, 140), the activation of the transcription factor cyclic adenosine 3’,5’-monophosphate (cAMP)-responsive element binding protein (CREB) (141) and the direct inactivation of human caspase-9 (142).
With the caveat of the functional intricacies present in the interaction between AKT and both of the mTOR complexes, the observation that AKT can regulate mTOR activity opened the possibility that AKT can indirectly regulate protein translation (Figure 1.5) by either enhancing ribosomal-dependent protein synthesis through the activation of p70S6 kinase (143, 144) and through the mTOR-dependent inactivation of the translation repressor 4E-BP1. In contrast with the fundamental role of AKT in conventional T cell function, results from recent studies are consistent with the negative effect of AKT signaling in the development and suppressor function of Tregs.

1.7 The role of AKT in regulatory T cell lymphocytes

1.7.1 Inhibitory effect of AKT on Tregs

In 2008 Sauer et al. (145) reported that the PI3K/AKT activity antagonized FoxP3 expression in murine T cells. The involvement of the AKT pathway was further suggested in FoxP3 expression when a constitutively active AKT repressed the TGF-β induction of FoxP3 in a rapamycin-sensitive manner. In addition, the combined inhibition of both PI3K and mTOR greatly augmented FoxP3 induction in CD4+ T cells (145). Only a moderate direct inhibition of AKT was sufficient to increase FoxP3 expression in T cells, conveying that the blockage of AKT itself promoted FoxP3 expression rather than an upstream regulator. In a comparison of PI3K/mTOR inhibitor-induced cells to freshly isolated Treg cells, more than half of the transcripts specifically regulated in Treg cells were similarly regulated in FoxP3-induced cells via PI3K/mTOR inhibition, suggesting a substantial association between suppression of the PI3K/AKT pathway, and \emph{de novo}
expression of FoxP3 and Treg signature in mouse CD4+ T cells (145). The involvement of AKT in Treg development was further investigated by Haxhinasto (146), who reported that if FoxP3 expression was already established in T cells, the constitutive activation of AKT did not significantly decrease its expression. The authors also investigated the role of AKT in the development of nTregs and concluded that, like iTregs, activation of AKT significantly impaired thymic CD4+FoxP3+ nTreg development without altering positive selection of conventional CD4+ and CD8+ T cells. These results placed AKT at a nexus of signaling pathways with broad impact on the onset of Treg specification in thymus as well as on the differentiation of peripheral iTregs.

In addition to the critical role of AKT on Treg development, recent reports established the negative impact of AKT on the suppressive function of human nTregs. Crellin et al. (147) investigated whether hyporesponsiveness and lack of cytokine production in Tregs compared to conventional T cells was the result of altered TCR signaling. The authors determined that CD4+CD25- T cells and CD4+CD25+ Tregs had equivalent levels of ERK1/2 and p38 phosphorylation upon TCR stimulation. However, CD4+CD25+ Treg cells displayed a consistent defect in phosphorylation of AKT at S473 and T308 as well as reduced phosphorylation of the AKT substrates FoxO and S6, suggesting a specific blockade of the TCR-induced AKT pathway. Moreover, constitutive activation of AKT in nTregs led to increased proliferation and concomitant abrogated suppressive capacity, further supporting the requirement of a “silent” AKT to sustain Treg function. In addition, enhanced AKT activity in CD4+CD25+ nTreg cells did not suppress expression of FoxP3, CTLA-4 or CD25 which indicated that the loss of function
was not related to a phenotypic change (147). In conclusion, these seminal studies demonstrated the connection between biological function and the altered AKT signaling pathway in CD4^+CD25^+ nTreg cells. Unlike the AKT requirement in the conventional T cell effector response, AKT activation in Tregs significantly impairs their development and function. In an effort to understand the mechanisms that regulate the transition from conventional T cells to iTregs, our group has recently reported that the rewiring of the TCR pathway occurring during the iTreg differentiation also results in the specific blockade of TCR-dependent AKT activation and has elucidated some of the signaling events involved in this altered TCR signaling (148).

1.7.2 The AKT network determines effector T cell sensitivity to Treg suppression

The AKT pathway is not only critical for Treg development and function, but is also an essential determinant of effector T cell sensitivity to Treg cell-mediated suppression. In several murine models of spontaneous autoimmunity, such as the non-obese diabetic (NOD) model of diabetes and the MRL/Mp model of systemic lupus erythematosus, Tregs function normally, but effector T cells are resistant to Treg-mediated suppression (149-151). In addition, several strains of mice with altered T cell intracellular signaling were also refractory to iTreg activity (152, 153). It was not until King et al.’s publication (154) that a potential mechanistic explanation for the effector T cell insensitivity to Tregs was found in the hyperactivation of the PI3K–AKT pathway. Concrete evidence for a causal relationship between AKT and effector T cell resistance was confirmed in a recent study by Wehrens et al. (44) in human patients with juvenile
idiopathic arthritis (JIA). Tregs from synovial fluid and peripheral blood of patients with JIA were fully functional when compared to healthy controls; however, at the local site of autoimmune inflammation, proliferation and cytokine production of effector T cells was poorly controlled by Tregs. These T cells isolated from the site of inflammation had increased levels of active AKT, and the pharmacological inhibition of AKT kinase activity rendered them susceptible to Treg-mediated suppression. These findings demonstrate that not only a weak Treg niche, but also a dysfunctional effector population may account for the attenuated suppressor activity and loss of immune homeostasis (43). Further evidence for the importance of AKT in the effector T cell interplay with Tregs can be found in the homeostatic control of the maturation of Ag-primed cytotoxic T cell (CTL) precursors to become CTL effector cells by a mechanism that involves the inhibition of AKT, but not of STAT5 or Zap70 (155).

1.8 The role of ion channels in T cells

Ion channels are essential to proper T cell function as they are intimately involved in signal transduction, maintenance of membrane potential, cell motility and cell volume in T cells. Potassium ion channels (K⁺ channels) in T cells regulate the ability to maintain negative membrane potential during TCR induced Ca²⁺ flux and without them, Ca²⁺ flux would cease, resulting in a halt to TCR signaling. There are two common types of K⁺ channels that contribute significantly to the ionic environment in T cells: Kv1.3 and KCa3.1. Upon TCR ligation with MHC/antigen complex, IP₃ diffuses through the cytosol and binds to its receptor (IP₃R) on the endoplasmic reticulum. IP₃R is a ligand gated Ca²⁺ channel that will release Ca²⁺ into the cytosol of the cell upon activation (156). Ca²⁺
released from stores in the ER results in the opening of plasma membrane Ca\(^{2+}\) release-activated Ca\(^{2+}\) (CRAC) ion channels, causing Ca\(^{2+}\) to influx into the cell from the external fluid (157, 158). The rise in intracellular Ca\(^{2+}\) activates calcineurin, which, in turn, activates NF-AT thus leading to the production of pro-inflammatory cytokines, and to T cell differentiation and proliferation (126). Negative membrane potential necessary for regulation of the magnitude of TCR-induced Ca\(^{2+}\) as well as Ca\(^{2+}\) influx (159) is maintained by K\(^+\) channels sensitive to changes in membrane potential (Kv1.3) or Ca\(^{2+}\) concentration (KCa3.1) (160). Activation of K\(^+\) ion channels stabilizes TCR-induced signaling by stabilizing Ca\(^{2+}\) influx (Figure 1.6) (161), thus making K\(^+\) ion channels attractive candidates for pharmacological intervention. Specific inhibitors of Kv1.3 (Margatoxin) and KCa3.1 (TRAM34) have been described in the literature (161). Kv1.3 expression on specific T cell subsets has been linked to autoimmune disease.
Figure 1.6: TCR induced Ca\textsuperscript{2+} signaling and ion exchange.

Upon TCR engagement with MHC/antigen complex, phospholipase C gamma (PLC-\(\gamma\)) generates the second messenger IP\(_3\), inducing the release of Ca\textsuperscript{2+} from the endoplasmic reticulum. Increased cytosolic Ca\textsuperscript{2+} induces opening of CRAC channels in the plasma membrane, thus allowing a Ca\textsuperscript{2+} influx. Kv1.3 and KCa3.1 channels open allowing K\textsuperscript{+} efflux which contributes to the negative membrane potential that is the driving force for Ca\textsuperscript{2+} influx. Reproduced with permission from (161).
An effector T cell response is generated upon antigen encounter. After the threat or pathogen has been eliminated in the body, a small percentage of the effector T cells differentiate into memory T cells that can respond very rapidly to another challenge. Two main types of memory CD4⁺ T cells are generated: central memory T cells (T_{CM}) and effector memory T cells (T_{EM}). T_{CM} have little to no effector function, but can readily proliferate and differentiate to effector T cells when challenged with antigen. T_{EM} are protective memory T cells that display characteristic sets of chemokine receptors and adhesion molecules that facilitate homing to inflamed tissues where they exert immediate effector function (162). Chronically activated T_{EM} cells have been implicated in tissue damage associated with several autoimmune diseases, including MS (163), RA, asthma and type-I diabetes mellitus (164). Recent studies suggest that, when activated, T_{EM} exhibit a specific change in K⁺ ion channel expression that does not occur in other T cells types (163, 165). Specific stimulation of either naïve or T_{CM} cells results in only modest increases in expression of Kv1.3 channels, whereas KCa3.1 channel numbers were dramatically up-regulated upon stimulation of T_{EM} cells. However, T_{EM} activation leads to a significant increase of Kv1.3 channels without any change in levels of KCa3.1 (162). Up-regulation of specific K⁺ channels also coincides with increased proliferative ability of the T_{EM}, thus it is reasonable to believe that targeting Kv1.3 in the diseases where T_{EM} are provoking the pathology may be beneficial. These findings led several groups to investigate Kv1.3 channels in T_{EM} as a therapeutic target for treating autoimmune diseases like MS (166), however investigation into Kv1.3 channel manipulation should include special consideration of Kv1.3 expression levels on Tregs as well due to the
importance of Kv1.3 in iTreg signaling mechanisms. Though inhibiting Kv1.3 on the pathogenic T<sub>EM</sub> has been suggested to control the disease severity, Kv1.3 inhibition on Tregs could lead to exacerbation of the disease. Therefore, understanding the role of the K<sup>+</sup> ion channels in Tregs will be crucial to predicting and understanding the effects of this clinical trial on Treg function.

1.9 IL-2 Signaling in T cells

IL-2 is a cytokine primarily produced by CD4<sup>+</sup> T cells following their activation by antigen that is widely considered to be a key cytokine in the T-cell dependent immune response (167, 168). IL-2 can promote T cell growth, augment NK cell cytolytic activity, prompt differentiation of Tregs and mediate activation-induced cell death in T cells (167). The IL-2 receptor consists of three different chains α, β and γc (Figure 1.7a) (168). IL-2Rα chain (CD25) is expressed on activated T cells and binds to IL-2 alone with fairly low affinity. The IL-2Rβ (CD122) and the IL-2Rγ (common cytokine receptor γ chain) form a complex that binds IL-2 with intermediate affinity (169). When all three IL-2R chains are expressed in a cell the IL-2R becomes a high-affinity receptor and initiates strong IL-2R signaling. Expression of the high-affinity IL-2R is critical for T cells responses to low concentrations of IL-2 (170). CD25 expression is absent on naive and memory T cells but is induced after antigen activation. IL-2Rβ is constitutively expressed by NK, NKT, and memory CD8<sup>+</sup> T cells but is also induced on naive T cells after antigen activation while γc is constitutively expressed by all lymphoid cells (170). Once the high-affinity IL-2R is induced by antigen, IL-2R signaling upregulates the expression of CD25...
in part through Stat5-dependent regulation of *IL-2ra* transcription (171). This process represents a mechanism to maintain expression of high-affinity IL-2R and sustain IL-2 signaling. Upon ligation of IL-2 to its receptor, a series of signaling cascades are activated which include phosphorylation of signal transducer and activator of transcription 5 (STAT5) which translocates to the nucleus of the cell where it will regulate transcription; activation of PI3K which causes downstream activation of AKT; and phosphorylation of mitogen-activated protein kinase (MAPK) – all of which will lead to growth and survival of the T cell along with transcriptional regulation and effector T cell differentiation (Figure 1.7b).
Figure 1.7: IL-2 Signaling.

(a) IL-2R is composed of three subunits IL-2Rα (CD25), IL-2Rβ (CD122), and IL-2R γc, the combination of which creates a high affinity receptor. (b) Upon IL-2 binding to the receptor it will activate a signaling cascade leading to STAT5, PI3K and MAPK activation subsequently inducing growth, survival, transcriptional regulation and effector differentiation in the T cell. Reproduced with permission from (168).
IL-2 is required, not only for iTreg and nTreg development, but maintenance as well (20). Mice deficient in IL-2R develop a lethal autoimmune disease secondary to failed thymic development and impaired peripheral homeostasis of nTregs (172). The constitutive expression of the high-affinity IL-2R as detected by expression level of CD25 is used to characterize most Tregs. IL-2 signaling in Treg cells differs from conventional T cells in that Treg cells essentially fail to activate the PI3K-AKT pathway because of high PTEN protein expression (173, 174). Indeed, though Tregs express high-affinity form of the IL-2R, increased levels of PTEN at least partially account for the failure of Tregs to undergo IL-2 dependent proliferation in vitro (174). Additionally, IL-2 signaling through STAT5 activation has been found to promote FoxP3 expression in iTregs (175). Understanding IL-2 signaling mechanisms will undoubtedly prove to be a crucial step in discerning critical signaling pathways in iTreg development.

1.10 TGF-β Signaling in T cells

Transforming growth factor-β (TGF-β) is a cytokine conserved across all leukocyte lineages, including lymphocytes, macrophages and dendritic cells, the production of which effects the differentiation, proliferation and activation of the immune cells (176, 177). TGF-β plays a pivotal role in regulating the immune response. Signaling through the TGF-β pathway will inhibit T and B cell proliferation and induce apoptosis as well as prevent T_{H}1, T_{H}2, or CTL differentiation. TGF-β can bind to both type I and II transmembrane serine/threonine kinase receptors (Figure 1.8) (178). However TGF-β signaling is a linear pathway initiated through TGF-βRII only, which will, in turn,
activate TGF-βRI leading to the phosphorylation of intracellular SMAD proteins. There are eight known SMAD proteins: receptor associated SMADs (R-SMADs1, 2, 3, 5, and 8), one common SMAD (Co-SMAD4), and two inhibitory SMADs (I-SMAD6 and 7). R-SMADs are sequestered in the cytoplasm in the absence of TGF-β signaling and are presented to TGF-βRI by scaffolding proteins such as SMAD anchor for receptor activation (SARA) (179), hepatic growth factor-regulated tyrosine kinase substrate (Hrs) (180), and disabled homolog 2 (Dab-2) (181). Once TGF-βRII activates TGF-βRI the R-SMADs will become phosphorylated and will associate with Co-SMAD4. This R-SMAD/Co-SMAD complex translocates into the nucleus where it will bind to the SMAD-binding element (SBE) in the genome inducing transcriptional regulation (182). It has recently been suggested that TGF-β plays an important role in the plasticity seen between iTreg and T_{H17} differentiation. T_{H17} lineage has been identified as a subset that produce IL-17 and are generated in response to TGF-β and IL-23 or IL-6 signaling (183). However, it has been found that TGF-β signaling along with IL-2 will induce the development of iTregs (20, 21, 184, 185). In more recent studies, it has been shown that SMAD3 binds to an enhancer of the FoxP3 gene, suggesting a possible role of TGF-β in iTreg differentiation (186, 187). Production of both inflammatory and anti-inflammatory cells through TGF-β signaling is believed to be accomplished through the influence of downstream cytokine signaling with divergent outcomes. Tight regulation of the additional signaling pathways on TGF-β signaling allows the interplay between the two pathways generating reciprocal effects. Conversely, SMAD-independent signaling may be regulating the differentiation of the proinflammatory T_{H17} cells whereas SMAD3
dependent signaling is regulating iTreg development. Nonetheless, the definitive role of TGF-β in the induction of iTregs and the cross-talk between TGF-β and other signaling pathways will be vital in elucidating its role in the development of iTregs.
Figure 1.8: TGF-β Signaling
TGF-β binding to TGF-βR leads to SMAD2 or SMAD3 activation/phosphorylation. Once phosphorylated, SMAD2/SMAD3 binds to the co-SMAD4 and translocates from the cytosol to the nucleus where it regulates transcription by binding to DNA. SMAD2/SMAD3 transcriptional regulation leads to differentiation, growth inhibition, deposition of extracellular matrix and apoptosis. Reproduced with permission from (178), Copyright Massachusetts Medical Society.
1.11 Overview of thesis

In this thesis, we will use a novel cell culture system that mimics human iTreg development to analyze the hypothesis that molecular reorganization of the TCR signaling network is critical for the differentiation of human peripheral CD4⁺ T cells into iTregs. In Chapter 2, we present the details of the materials and methods used throughout the thesis. Chapter 3 investigates the hypothesis that primary human CD4⁺ T cells will differentiate \textit{ex vivo} into iTregs if cultured in the presence of anti-CD3, IL-2 and TGF-β. The overall goal is to generate a substantial number of human iTregs in order to use them as a therapeutic treatment for human disease and additionally to generate and expand stable iTregs to study the molecular mechanism that regulate their development. As iTreg development requires the presence of IL-2, TGF-β and CD3 stimulation, we expect to recapitulate this developmental milieu in culture. In Chapter 4, we will test the hypothesis that the differentiation of primary human T cells to suppressor iTregs involves the relocation of key proximal TCR signaling elements to the highly active IL-2R pathway. IL-2 is needed for iTreg development, however the signaling mechanisms behind this phenomenon and its cross talk to the TCR signaling pathway in human iTregs are unknown. Preliminary studies found that upon TCR activation there was no increase in phosphorylation of the Src-family kinase Lck suggesting a substantial rewiring of the TCR signaling pathway in iTregs. In Chapter 5 we will examine the reorganization of proximal TCR events during iTreg differentiation. Previously, it had been shown that AKT is inactive in regulatory T cells, and that this blockage of AKT activity occurs for Treg function. However it had not been determined what is causing the blockade of AKT activity. We will address two hypotheses, the first one that there is differential regulation
of phosphatases and kinases leading to blockage of AKT activity in iTregs. Moreover, iTregs need TGF-β signaling to induce FoxP3 expression; however TGF-β signaling may lead to apoptosis in the T cells. We hypothesize that AKT overexpression results in AKT-SMAD3 association, decreasing SMAD3’s ability to regulate transcription. We expect that AKT is regulating the balance of SMAD3 activity where it is need early during iTreg development, however if overly active can cause apoptosis in the cells. In Chapter 6 we will discuss the results found throughout this thesis and discuss potential future applications. This work provides a novel insight into the signaling mechanisms of iTreg development and has shed light on some potential targets for the use of iTregs as a therapy for the treatment of human disease.
Chapter 2: MATERIALS AND METHODS

2.1 Cell isolation, culture conditions, and sorting.

The generation of human iTregs _ex vivo_ has been described in detail in our papers elsewhere (148, 188). Briefly, human peripheral blood mononuclear cells (PBMC) from fresh buffy coats of healthy, anonymous blood donors (obtained from the Kentucky Blood Center, Lexington, KY) were isolated by centrifugation over a Ficoll-Hypaque gradient at 500 rcf for 30 min with no brake. CD4\(^+\) T cells were purified by negative selection (StemCell Technologies kit) and CD25\(^-\) cells were further selected (Miltenyi) following manufacturer’s instructions. CD4\(^+\)CD25\(^-\) T cells were cultured at a concentration of 2-2.5x10\(^6\)/mL in RPMI-1640 supplemented with 2.06 mM Glutamax-I and 25 mM HEPES, 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin, 50μM β-mercaptoethanol (2-ME), IL-2 (5 ng/mL, eBioscience), coated anti-CD3 antibody clone OKT3 (1 μg/mL, BioXCell) and TGFβ (2 ng/mL, Invitrogen). Cells were maintained in culture for 5-7 days with a change of medium on the third day. Naïve (CD45RA\(^+\)CD25\(^-\)), memory (CD45RA\(^-\)CD25\(^-\)) and iTreg (CD45RA\(^-\)CD25\(^{high}\)CD127\(^{low}\)) cells were sorted using a Cytomation MoFlo (Beckman Coulter) high speed sorter (University of Kentucky Cytometry Core Facility) and antibodies against CD45RA, CD127, CD25 (Miltenyi). Cells were also analyzed directly from culture using antibodies against CD25, CD45RA, CD127, GITR, FoxP3 and CTLA4 (BD). The acquisition of Treg-like phenotype (high expression of CD25, FoxP3, CTLA4, GITR and low expression or absence of CD45RA and CD127) is concomitant with cells displaying a strong capacity to suppress the proliferation of CD4\(^+\) T cells (148) which we confirmed.
in the present study. Between 20% and 35% of original CD4+CD25− T cells reproducibly develop to the iTreg phenotype. For optimization of iTregs numbers the CD4+ T cells were cultured in AIMV (GIBCO), anti-CD3 (1µg/mL), IL-2 (5 ng/mL) and TGF-β (2 ng/mL).

2.2 Suppression Assay

Purified CD4+CD25− cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) (Molecular Probes) at a final concentration of 5µM in PBS with 0.1% BSA. CFSE labeled cells (10^5 per well) were cultured with CD3-CD28 beads (Inspector kit, Miltenyi) in the presence of sorted naïve, memory, effector T cells or iTregs (3x10^4 per well-ratio target CFSE labeled cells: effector cells of 3:1). After 5 days of co-culture, cells were harvested and the CFSE profile of the stained cells was analyzed by flow cytometry. The sequential dilution of CFSE fluorescence illustrates the number of cell divisions of target cells.

2.3 Flow Cytometry

All flow cytometry analysis was conducted on FACS Calibur (Becton Dickinson) using Cell Quest Pro Software (BD Bioscience) and further analyzed with FlowJo software (Tree Star, Inc.). For surface staining, 3X10^5 cells were washed in cold FACS buffer (PBS with 0.5% (w/v) bovine serum albumin (BSA) and 2 mM EDTA) and then incubated with saturating concentrations of appropriate antibodies for 15 minutes at 4°C. The cells were washed twice in cold FACS buffer before analysis. For intracellular staining, a FoxP3 staining kit was used (eBioscience) according to manufacturer’s
protocol. For experiments where the T cells were activated either with anti-CD3 or pervanadate, the cells were first rested for 6-7 hours at 4°C in RPMI without serum and then stimulated with anti-CD3 (0.5 mg/10^6 cells, BioXcell) and crosslinked with anti-mouse IgG (1.2 mg/10^6 cells, Jackson) or 2 mM pervanadate for 3 min at 37°C. Antibodies used for flow cytometry: CD45RA-FITC, CD127-APC, and CD25-PE (Miltenyi); FoxP3-APC and CTLA4-PE-Cy5 (BD); AKT-AF488 (Millipore); phosphorylated (P)-AKT S473, P-AKT T308, PTEN, P-PTEN, P-mTOR S2481, P-mTOR S2448 and mTOR (Cell Signaling); SMAD3 and P-SMAD3 (Abcam) as per manufacturer instructions. For detection of unconjugated primary antibodies, a secondary anti-Rabbit-AF647 (Invitrogen) was used. For detection of intracellular cytokines, secretion of cytokines was block by monensin, a GolgiStop protein transport inhibitor (BD kit) for 4 hours and cells were activated with 2.5 ng/mL phorbol myristate acetate (PMA) (Fisher) and 250 ng/mL ionomycin (MD Biomedical) for 2 hours at 37°C in an atmosphere containing 5% CO₂ before the staining. The same procedure as above was then used to stain the cells for surface and intracellular marker.

2.4 Western Blotting, immunoprecipitation and autoradiogram

Cells were collected from culture, washed with PBS and then in ice cold Radio-Immuno-Precipitation Assay (RIPA) buffer: 50mM Tris-HCl, pH 7.4; 150mM NaCl, 0.1% SDS, 0.5% Na-Deoxycholate and 1% Triton X-100, supplemented with 1mM PMSF and Halt Protease inhibitor cocktail (Pierce). Lystaes were either examined by Western Blot or used to immunoprecipitate proteins. To examine the interaction between
AKT and SMAD3, T cells were collected, washed with PBS and then lysed for immunoprecipitation in cold immunoprecipitation (IP) lysis buffer: 50mM Tris-HCl, pH 7.4; 150mM NaCl, 1% Nonidet P-40 and 0.5% n-dodecyl-b-D-maltoside. Lysis buffers were supplemented with 1mM PMSF and Halt protease inhibitor cocktail (Pierce). The lysates were pre-cleared using protein A/G PLUS agarose beads (Santa Cruz) then immunoprecipitated with either AKT antibody (Cell Signaling) or SMAD3 antibody (Abcam) and bound on agarose beads overnight at 4°C. After washing the pellet 5 times with IP lysis buffer, any bound AKT-SMAD3 was detected by Western blotting. For detection of Lck and CD122 interaction, lysates of 10^7 cells per condition were pre-cleared with Protein A/G agarose beads (Santa Cruz). Lck was immunoprecipitated from lysates by incubation for 2 h at 4 °C in Protein-A/G agarose beads precoated with either anti-Lck antibody (Santa Cruz) or isotype-matched IgG control. After 5 washes with IP lysis buffer, beads were resuspended in SDS-PAGE sample buffer, boiled for 3 min and analyzed by Western blot. Proteins from whole cell lysates or from immunoprecipitates were resolved on 10% or 12% gels by SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membrane, blocked for 1 hour in 2% FBS in TTBS at 37 °C and incubated overnight in primary antibody (1 μg/mL or the concentration recommended by manufacturer) at 4°C. Membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibody for 30 min. at room temperature. Labeled proteins were visualized by chemiluminescence (Pierce ECL Western blotting substrate, Thermo Scientific). Western blots were analyzed with the following antibodies: anti-P-Tyr (clone PY99), anti-Lck (clone 3A5), anti-Zap70, anti-LAT, anti-AKT, anti-ERK 1/2,
anti-CD122 (IL2-R β subunit), anti-Grb-2 and anti-GAPDH-HRP (Santa Cruz); anti-P-Src (Y416); anti-Zap70 and anti-P-Zap70 (Y493); anti-STAT5 and anti-P-STAT5 (Y694); anti-P-LAT (Y191); anti-P-AKT (Ser473); anti-P-ERK 1/2 (Thr202/Tyr204), anti-AKT, anti-Plcγ, anti-P-PTEN, anti-PTEN, and anti-mTOR (Cell Signaling). Kv1.3 antibody (APC-002) was purchased from Alomone labs. For detection of unconjugated primary antibodies, a secondary anti-rabbit-HRP and/or an anti-mouse-HRP (Jackson) were used. Densitometric analysis with the ImageQuant v.5.1 software (Molecular Dynamics) was used to quantify the intensity of labeling. Pixel densities for each band were normalized within the same experiment and autoradiography exposure in order to reduce inherent variability of experiments and ECL exposure times.

2.5 Ca\(^{2+}\) influx in T cells

Human CD4\(^+\) T cells were obtained from iTreg polarizing culture (anti-CD3, IL-2, TFG-β) and washed with PBS. The cells were then stained with 1 μl/mL of 10 X 10\(^6\) cells Indo-1 dye (Invitrogen), which is a dye that will fluoresce at two different wavelengths depending on whether (400nm) or not (475 nm) it is bound to Ca\(^{2+}\), and incubated for 30 minutes at 37\(^\circ\)C. Indo-1 stained cells were washed and then were treated with antibody against CD25 (Miltenyi), CD45RA (Miltenyi) and either biotinylated-CD3 (Miltenyi) alone or biotinylated-CD3 clone OKT3 and biotinylated-CD28 clone CD28.2 (Miltenyi) on ice for 15 min. After incubation with antibodies the cells were washed with PBS and treated with RPMI containing either 60 nM Margatoxin (Kv1.3 inhibitor) (Sigma), 60 nM TRAM34 (KCa3.1 inhibitor) (ENZO), or media alone at 37\(^\circ\)C degrees in
the presence of 5% CO₂. Cells were washed and resuspended in hank’s balances salt solution (HBSS) (GIBCO). A baseline reading of Ca²⁺ flux was obtained by flow cytometer. Immediately following the addition of 5 mg/mL avidin (Invitrogen) to induce TCR signaling by crosslinking biotinylated antibodies, Ca²⁺ influx was measured again by reading both Indo-1 wavelengths for 5 minutes in a flow cytometer (Figure 2.1). T cell populations were distinguished by their expression of CD25 and CD45RA, naïve cells by their expression of CD45RA⁺CD25⁻ and iTregs cells by their expression of CD45RA⁻CD25⁵⁻. The Ca²⁺ flux demonstrated in this dissertation is due to extracellular Ca²⁺ added to samples. Analysis of data was determined by the percentage inhibition, Ca²⁺ flux peak values were calculated for each population in RPMI only and set equal to 100 percent. Ca²⁺ flux peak values in the presence of Margatoxin or TRAM34 were subtracted from Ca²⁺ flux peak values in the presence of RPMI or DMF only. This difference was labeled as the percentage inhibition.
Figure 2.1: Measuring Ca\textsuperscript{2+} influx

Experimental procedure for measuring TCR-induced Ca\textsuperscript{2+} flux. Primary human CD4\textsuperscript{+} T cells were cultured for 5-6 days in iTreg polarizing media (anti-CD3, IL-2 and TGF-\(\beta\)), stained with Indo-1 AM calcium dye and incubated for 30 min at 37°C. Indo-1 loaded cells were washed and then labeled with biotinylated anti-CD3, biotinylated CD28, CD25 and CD45RA. Baseline readings were taken for 30 seconds and avidin (5 mg/mL) was added to induce CD3 and CD28 crosslinking. Samples were then read for 5 minutes. Indo-1 dye fluoresced at different wavelengths when bound or unbound to Ca\textsuperscript{2+} thus allowing for investigation of intracellular Ca\textsuperscript{2+} levels within each T cell population.
2.6 Electrophysiology

We used the automated IonWorks HT high-throughput patch-clamping system (Essen Instruments, Ann Arbor, MI) as a screening platform to determine Kv1.3 activity profiles in different T cell subsets and culture conditions. We used 100 mM K⁺-D-gluconic acid, 50 mM KCl, 3mM MgCl₂ and 5mM EGTA pH 7.3 as intracellular recording solution in the high-throughput patch-clamping screening. We followed the protocols and conditions as detailed elsewhere (189). Briefly, isolated lymphocytes in suspension (5 × 10⁵/mL) were dispensed into a 384-well patch-plate. The bottom of each well contained a micropore with a diameter of 1.6-1.8 μm allowing a single lymphocyte to be suctioned and positioned over the hole in each well. After electrical access to the cytoplasm was obtained, a depolarizing voltage step function from -80 to +40 mV was applied across the cell membrane. This depolarization opened voltage-gated ion channels, and the resulting whole-cell current through the plasma membrane was recorded. While the same cell was still attached, a specific blocker of Kv1.3 channels was added to each microwell. A second depolarizing step was applied to the cell, and the resulting whole-cell current was recorded. The Kv1.3-specific current was defined as the difference between the pre-blocker and post-blocker electrical currents (Figure 2.2).
Figure 2.2A-C
Figure 2.2: High-throughput method for measuring Kv1.3 ion channel activity

(A) Isolated lymphocytes in suspension ($5 \times 10^5$ mL) were dispensed into a 384-well patch-plate. The bottom of each well contained a micropore with a diameter of 1.6-1.8 μm allowing a single lymphocyte to be suctioned and positioned over the hole in each well. (B) After electrical access to the cytoplasm was obtained, a depolarizing voltage step function from -80 to +40 mV was applied across the cell membrane. This depolarization opened voltage-gated ion channels, and the resulting whole-cell current through the plasma membrane was recorded. (C) While the same cell was still attached, a specific blocker of Kv1.3 channels was added to each microwell. A second depolarizing step was applied to the cell, and the resulting whole-cell current was recorded. The Kv1.3-specific current was defined as the difference between the (B) pre-blocker and (C) post-blocker electrical currents. High-throughput patch clamp assay afforded us the ability to read the activity of Kv1.3 ion channels within 100s of lymphocytes simultaneously. Figure modified from (189).
2.7 Gene Expression

Transcription of the AKT, PTEN, mTOR, and SMAD3 genes was quantified from CD25− T cells, effector T cells, and iTregs. FOXP3 (as a positive control) and GAPDH (housekeeping gene) were assessed as control. Total RNA was extracted from 5-7 X 10⁶ sorted cells. cDNA was obtained by reverse transcription and used as a template in a microarray analysis with the Affymetrix GeneChip System (Human Genome U133 Plus 2.0 Array microchip) at the UK Microarray Facility. Results represent the comparative mean value of triplicates from three different donors relative to CD25− samples; bars represent the experimental range of the triplicates. Pertinent results were validated with real-time PCR.

2.8 Immunofluorescence Microscopy

Cells were first rested for 6-7 hours on ice and then stimulated with anti-CD3 (0.5 mg/10⁶ cells, BioXcell) crosslinked with anti-mouse IgG-Rhodamine Red-X (1.2 mg/10⁶ cells, Invitrogen) for 3 min at 37°C. Cells were washed in PBS and fixed in 3.7% paraformaldehyde in PBS, washed in PBS, permeabilized for 2 minutes in PBS containing 0.5% TritonX-100, then washed in PBS and incubated in blocking solution (PBS 5% BSA with goat serum) for 1 hour. Cells were then washed in PBS and incubated with anti-AKT-AF488 (Millipore). Cells were washed 3 times in PBS, cytopspinned for 3 minutes at 800RPM low acceleration on a Shandon Cytospin 4 (Thermo Electron Corporation) to flatten cells on slides, and then coverslips were mounted onto slides using ProLong Gold Antifade reagent containing DAPI (Invitrogen). Appropriate controls were conducted to demonstrate the specificity of both primary and
secondary antibody labeling under all conditions. Deconvolution microscopy was conducted using a Carl Zeiss Imager.Z1 microscope and AxioVision Rel 4.8 software (Carl Zeiss Worldwide). A Z-series of focal planes were digitally imaged and deconvolved with the AxioVision constrained iterative algorithm (190). The process removes out-of-focus light and reassigns it to its point of origin to generate high resolution images. Multiple images containing several cells were collected and representative cells are shown. All images were digitally processed for presentation using ImageJ (191). Images shown are deconvolved and represent single Z-sections. For quantification of images, the colocalization highlighter plugin from ImageJ was used to determine the colocalization of AKT-CD3 (Institut Jacques Monod, Service Imagerie, Paris). AKT-CD3 colocalization was considered to be membrane bound AKT. Membrane-bound AKT was expressed as a percentage of total AKT from each cell. To examine the interaction between AKT and SMAD3, cells were collected from culture and then washed with PBS, following the same protocol as above for fixation and permeabilization. The cells were stained using primary antibodies against anti-AKT-AF488 (Millipore) and anti-SMAD3 (abcam). For detection of unconjugated primary antibodies, a secondary anti-Rabbit-AF647 (Invitrogen) was used. The cells were then imaged using the same deconvolution steps as above.

2.9 Transfections and Luciferase reporter assays

All transfections were performed using the AMAXA nucleofector system (LONZA) following the manufacturer’s instructions for stimulated human T cells program T-020 for conventional T cells and T-002 for iTregs. Cells were washed with
PBS and then transfected using 5 μg of pGL3 Basic (CAGA)_{12} MLP Luciferase (graciously donated by Dr. Ten Dijke (192)). Values were normalized with the renilla luciferase activity expressed from pGLRP (graciously provided by Dr. Michael Kilgore, University of Kentucky). Cells were collected after 24 hours in culture and luciferase activity was measured using Dual-Luciferase reporter system (Promega) and Lumat LB 9507 luminometer (Berthold Technologies). Relative luciferase units are luciferase intensity/renilla intensity for each cell type. Luciferase values shown are representative of transfection experiments performed in triplicate in at least three independent experiments with different donors.

2.10 Lipid Raft Isolation

Purified T cells were subjected to a 5-80% sucrose gradient centrifugation. Briefly, T cells were resuspended in MNE buffer (25mM MES (Sigma), 5mM EDTA (BioExpress), 0.15M NaCl (Sigma) and in ddH$_2$O) and incubated on ice for 15-30 min. The cells were then lysed using a cold dounce homogenizer with lysis buffer (MNE, Triton X-100 (Fisher) and Halt protease inhibitor cocktail (Pierce)). The lysate was mixed with 80% sucrose (Fisher) and a 30% sucrose layer was added on top slowly careful to not disturb the two layers in ultracentrifugation tubes (Beckman). Lastly, a 5% sucrose layer was added on top without disturbing the other layers. Ultracentrifugation was performed on the gradient spinning overnight at 35,000 rpm, 4°C with no brake using Beckman Optima LE-80K Ultracentrifuge with SW55T rotor. After centrifugation, layers of 200 μl fraction were taken, RIPA buffer was added to each sample and incubated for 30 minutes on ice. 500 μl of methanol was then added to each fraction and vortexed.
After vortexing, 250 µl of chloroform was added and the samples were vortexed again. Lastly, 500 µl of ddH₂O was added, samples were vortexed and then centrifuged for 5 min at maximum speed. The supernatant was removed and the pellet containing protein precipitates was washed with methanol, vortexed and centrifuged for 8 min at max speed. The supernatant was then removed and the pellet was allowed to dry. Once dry, the pellet was resuspended in sample buffer and analyzed by Western blot analysis as described above.

2.11 Statistical Analysis

The Kruskal-Wallis ANOVA test was used to determine differences in peak currents of Kv1.3 among multiple groups. Statistical comparisons of current distributions between two samples were performed using the Kolmogorov-Smirnov test. Differences were considered significant when \( p \) values were <0.01. The Repeated Measures ANOVA with a Student Newman-Keuls multiple comparison post-hoc analysis was used to determine differences in protein expression among three different T cell subsets. Differences between conventional and Tregs were analyzed using a two-tailed, paired student’s t-test. Differences were considered significant when \( p<0.05 \).
Chapter 3: Differentiation of Human iTregs \textit{ex vivo}

3.1 Introduction

Numerous diseases are caused by the dysregulation of regulatory T cells (17). Hence, understanding the precise mechanisms involved in their development, activation and suppressor function could provide potential insight for therapeutic use. The population of regulatory T cells known as iTregs has recently been shown to be the more prevalent source of circulating Tregs (25). Key evidence for the peripheral generation of Tregs comes from Vukmanovic-Stejic et al. in which they found human Tregs to be a rapidly proliferating population compared with naïve or memory T cells. However these cells were short lived and underwent apoptosis at a relatively rapid rate compared to naïve or memory T cells (24, 25). They also found that the number and function of CD4^{+}CD25^{+}FoxP3^{+} Tregs are maintained in humans even after the age of 70 (23). Hence, nTregs, or expansion of these cells, cannot explain the lifelong existence of human Tregs, especially when thymic function is reduced after puberty in man and the number of Tregs does not decrease with age. Therefore, they suggest that the majority of peripheral Tregs are iTregs (24, 193). Although murine models of colitis (194), type I diabetes (42), RA and graft versus host disease (195) have shown that injecting Tregs to be a beneficial therapeutic, there are fundamental differences between mouse and human Treg biologies which need to be elucidated before their clinical use (188). The lack of sufficient number, purity, stability and homing specificity to therapeutic Tregs necessitates an experimental platform to optimize conditions for their \textit{ex vivo} expansion (196).
Therefore, our lab has generated a novel cell culture model that mimics \textit{in vivo} human iTreg development. This innovative system allows for the differentiation and expansion of iTregs \textit{ex vivo} from an individual donor after 5-6 days in culture, along with the ability to simultaneously compare iTregs to naïve, memory and effector T cell populations within the same donor. Using this experimental platform, we characterize the day-to-day kinetics of iTreg development and the phenotypical analysis of the cell surface and intracellular T cell markers expressed on human iTregs, investigate the iTreg suppressor capacity by suppression assay through cell contact and soluble factors, examine cytokine production and determine the extent of iTreg proliferation.
3.2 Results

3.2.1 Experimental procedure and kinetics of iTreg development

In order to investigate the generation of human iTregs *ex vivo*, we obtained buffy coats from the Kentucky Blood Center from anonymous donors. PBMCs were isolated by centrifugation over a Ficoll-Hypaque gradient. The CD4⁺ T cells were then purified by negative selection and CD25⁻ cells were further selected using magnet separation (Figure 3.1). The CD4⁺CD25⁻ T cells were cultured in iTreg polarizing media that contained anti-CD3, IL-2 and TGF-β for 5-6 days. The iTregs were then purified using FACS. Figure 3.1 illustrates the experimental procedure used to purify the iTreg population. Monitoring iTreg development overtime in our culture system found that at day 0 in culture, most the T cells are CD25⁻FoxP3⁻CTLA4⁻ (Figure 3.2). After one day of culture, CD4⁺ T cells start to express low levels of CD25 and CTLA4, with a few of the cells expressing FoxP3 (Figure 3.2). As cells progressively develop into iTregs they express higher levels of CD25, CTLA4 and FoxP3 until 20-35% of T cells have differentiated into iTregs expressing high levels of CD25, CTLA4, and FoxP3, and are CD45RA negative (Figure 3.2) at the end of five days in culture. Additionally, we have been able to optimize on cell culture conditions to generate approximately 80% of the CD4⁺CD25⁻ cells into iTregs (Figure 3.3). To do this we cultured our CD4⁺CD25⁻ T cells in AIM V media, a proprietary serum free formulation, optimized for proliferation of T cells. Therefore, this novel cell culture system enables the development of a substantial number of regulatory T cells. If otherwise noted, we will be using standard iTreg polarizing conditions along this dissertation.
Figure 3.1: Schematic of experimental procedure.
PMBCs are separated out of human peripheral blood via gradient centrifugation before magnetic negative selection of CD4+CD25- T cells. After five to six days in culture, cells undergo FACS and are co-incubated with heterologous CFSE labeled target cells to measure suppressor activity. Reproduced with permission from (188).
Figure 3.2: Kinetics of iTreg Development

Purified human primary CD4⁺CD25⁻ T cells are cultured in iTreg medium. An aliquot of cells is collected just after isolation (day 0) and at days 1, 3, 4, 5 of cell culture to monitor the progress of CD45RA, FoxP3, CTLA4 and CD25 markers. The iTreg profile corresponds to CD45RA⁻, FoxP3⁺, CTLA4⁺ and CD25⁺ (highlighted in the black image window). Reproduced with permission from (188).
Figure 3.3: Optimizing iTreg polarization
Purified human primary CD4 CD25 T cells are cultured in AIMV with anti-CD3, IL-2
and TGF-β for 5-6 days. An aliquot of cells is collected just after 5-6 days of cell culture
to monitor the progress of CD45RA, FoxP3, CTLA4 and CD25 markers. iTregs make up
about 80% of the cell culture.
3.2.2 iTregs originated from highly proliferating cells

After determining the kinetics of iTreg development we wanted to investigate the proliferative capacity of the T cells differentiating into iTregs. We have previously determined that iTregs start to develop at approximately day three in our culture system. We set out to determine if these cells were being induced from the proliferative cells or non-proliferative cells in culture. To do this, we purified human primary CD4⁺CD25⁻ T cells, stained them with CFSE, and then cultured them for 5-6 days in iTreg polarizing media with anti-CD3, IL-2 and TGF-β. After 5-6 days in culture, the cells were then analyzed by flow cytometry for the expression levels of CFSE. We found that T cells that differentiate into iTregs in our culture system are highly proliferative and undergo at least three or more cell divisions (96% of the cells divided) with a division index of 2.3 compared to CD4⁺ T cells who have only divided 22% with a division index of 0.4 after 5-6 days in iTreg polarizing culture (Figure 3.4) as seen by the decrease in CFSE upon each cell division. The division index represents the average number of cell divisions that a cell in the original population has undergone including the cells which never divided. These results indicate that during iTreg development, the T cells need to undergo a significant amount of proliferation before they can differentiate into iTregs.
Figure 3.4

**Figure 3.4: Proliferation of iTregs**
Purified human primary CD4\(^+\)CD25\(^-\) T cells were stained with CFSE and then cultured for 5-6 days in iTreg polarizing media with anti-CD3, IL-2 and TGF-β. After 5-6 days in culture the cells were then analyzed by flow cytometry using antibodies against CD25, and CD45RA. Proliferation in the cells was analyzed by CFSE dye dilution. iTregs are generated from highly proliferative cells that underwent at least three or more cell divisions (96% of the cell divided) with a division index of 2.3 compared to CD4\(^+\) T cells which have only divided 22% with a division index of 0.4. Figure is representative of three independent donors.
3.2.3 Phenotypic Analysis of human iTregs

Having investigated the developmental kinetics of iTregs along with showing that the cells that differentiate into iTregs are highly proliferative, we next wanted to conduct a more detailed examination of the iTreg phenotype generated in our cell culture system. To do this we cultured our cells for 5-6 days and then analyzed the expression of a complete panel of cell surface and intracellular markers. Our iTregs express higher levels of FoxP3 and CTLA4 when compared to naïve, memory and effector T cells (Figure 3.5). Also, iTregs exhibit high levels of GITR, which is present on activated T cells and is highly abundant on regulatory T cells (197) (Figure 3.5). CD127, the IL-7 receptor alpha chain had little to no expression on iTregs compared to conventional T cells. CD127 is usually present on effector T cells upon activation and has been found to inversely correlate with FoxP3 (198) (Figure 3.5). Additionally we detected CD86 expression in a subset of iTregs compared to naïve, memory and effector T cells. CD86 is the costimulatory molecule usually abundant on APC (Figure 3.6) that, upon ligation with its ligand, will initiate a signaling cascade necessary for T cell activation and survival (199). This expression of CD86 on iTregs is highly unique as it is not expressed on other T cell subsets; suggesting that iTregs could interact with other T cells through CD86. In our system, human naïve cells exhibit high levels of CD31, also known as platelet endothelial cell adhesion molecule (PECAM-1). CD31 is a protein member in the immunoglobulin superfamily likely involved in the migration of T cells (200); however human iTregs do not express CD31 (Figure 3.6) indicating a change in the pattern of migration in vivo. The chemokine (c-c motif) receptor 2 (CCR2) and CCR1 are also present on iTregs like their effector T cell counterparts (Figure 3.6) implicating that iTregs can traffic to the site of
inflammation. It has been reported that tumor leukocyte infiltrates express CCR1 (201). Moreover, human iTregs express CD58 or lymphocyte function-associated antigen 3 (LFA-3), which when bound to its ligand CD2 is thought to optimize immune recognition and T cell expansion (202) (Figure 3.6). CD58/CD2 ligation has been shown to strengthen the interaction between the T cells (203), which may be critical for iTreg interaction with its target reinforcing the connection between the two cells thus allowing iTregs to suppress their target cell. Lastly, CD30, a marker of activated T cells (204) was present on iTregs and has been proposed to be involved in the regulatory function of Tregs in vivo (205). These data suggest that the iTregs generated in our cell culture system are not only numerous in quantity but also possess a phenotype consistent with regulatory T cells.
Figure 3.5

Negatively selected human CD4⁺CD25⁻ T cells were stimulated in vitro. Based on the expression of CD25 and CD45RA, four cell populations can be distinguished after 5-6 days in culture: iTregs (CD25⁺CD45RA⁻, blue) and effector (CD25⁺CD45RA⁺, green), memory (CD25⁻CD45RA⁻, orange) and naïve (CD25⁻CD45RA⁺, red) T cells. The cell populations were then investigated for their expression of FoxP3, CTLA4, GITR and CD127. The iTreg population is CD25⁺, CD45RA⁻, FoxP3⁺, CTLA4⁺, GITR⁺ and CD127⁻/low.
Figure 3.6

 iTreg
 Effector
 Memory
 Naive

 Foxp3  CTLA-4  CD86  CD31

 iTreg
 Effector
 Memory
 Naive

 CCR2  CD58  CD30  CCR1
Figure 3.6: Detailed Phenotype of iTregs
Negatively selected human CD4⁺CD25⁻ T cells were stimulated in vitro. Based on the expression of CD25 and CD45RA, four cell populations can be distinguished after 5-6 days in culture: iTregs (CD25⁺⁺CD45RA⁻), memory (CD25⁺⁺CD45RA⁻), and naïve (CD25⁻⁻CD45RA⁻) T cells. The cell populations where then investigated for their expression of FoxP3, CTLA4, CD86, CD31, CCR2, CD58, CD30 and CCR1. iTregs were found to express FoxP3, CTLA4, CD86, CCR1, CCR2, CD58, and CD30, but to be CD31 negative.
3.2.4 Suppressive Ability of human iTregs

The human iTregs generated in our system express markers that have been found to be present on Tregs. However in order to identify iTregs a true regulatory T cell we wanted to investigate their ability to suppress the activation of effector cells. To test this, we cultured CD4+ T cells that had been stained with carboxyfluorescein succinimidyl ester (CFSE- a dye that will progressively dilute and halve in fluorescence with every division of the cell) with either purified iTregs, naïve or memory T cells for 5 days. The CFSE labeled/target cells were cultured at a ratio of 3 targets cells per 1 effector (iTreg/naïve/memory). After 5 days in culture we then analyzed the dilution of CFSE within the effector/target cells indicating the amount of proliferation in the cells. We found that, in the presence of naïve or memory cells, approximately 45% of the CFSE target cells proliferated (Figure 3.7). However, when the target cells were cultured with human iTregs, the proliferation was dramatically decreased to less than 15% of the CFSE target cells (Figure 3.7), suggesting that iTregs arrest the proliferation of effector T cells.

To investigate whether or not iTregs suppress through cell-to-cell contact or through soluble factors, we performed a Transwell suppression assay where the CFSE target cells and iTregs were separated by a 0.4 μm pore size membrane, thus preventing cell-to-cell contact. We determined that when the CFSE target cells were cultured at the same ratio as above with 3 target cells:1 iTreg, 61.8% of the CFSE target cells proliferated, suggesting that the mode of suppression may be through cell-to-cell contact (Figure 3.8). However, increasing the ratio of target cells to iTregs to 1:1 caused the proliferation of the CFSE target cells to decrease to about 37.6%. This effect was even more dramatic if
the ratio was changed to 1 CFSE target cell:3 iTregs (19.4%) implying that at higher quantities of cells human iTregs can suppress using soluble factors (Figure 3.8). Taken together these results indicated that human iTregs are in fact a suppressor T cell population which may utilize cell-to-cell contact and/or soluble factors to regulate effector T cell proliferation.
Figure 3.7: Suppression Assay

Purified CD4⁺ CFSE labeled effector cells (1 X 10⁵/well) are cultured with Treg suppression inspector beads in the presence of sorted naïve, memory or iTreg cells (3 X 10⁴/well) at a ratio of 3 target/CFSE cell:1 Treg. After five days, cells were harvested and the CFSE profile of the stained cells was analyzed by flow cytometry. The presence of iTreg cells completely abolished the proliferation of CD4⁺ T cells. Numbers are indicative of percentage of CFSE-labeled cells that have undergone division. Figure is representative of three independent donors. Reproduced with permission from (188).
Figure 3.8: Transwell Suppression Assay
Purified CD4\(^+\) CFSE labeled effector cells (1 X 10^5/well) are cultured with Treg suppression inspector beads in the presence of sorted iTreg cells (3 X 10^4/well) at three different ratios of target/CFSE cells:Treg (3:1, 1:1 and 1:3) in Transwell plates. iTregs along with the inspector beads were plated below the Transwell and the CFSE labeled cells were plated in the Transwell well. After five days, cells were harvested and the CFSE profile of the stained cells was analyzed by flow cytometry. The presence of iTReg cells completely abolished the proliferation of CD4\(^+\) T cells. Numbers are indicative of percentage of CFSE-labeled cells that have undergone division. Figure is representative of three independent donors.
3.2.5 Human iTregs produce IL-10

Having found that iTregs were secreting soluble factors that may suppress the proliferation of effector T cells, we wanted to identify potential soluble factor(s) involved. TGF-β and IL-10 are both anti-inflammatory cytokines that are produced by Tregs and have been identified to inhibit T cell proliferation (206). Due to the fact that recently it has been shown that TGF-β is more prevalent on the cell surface of Tregs rather than being a soluble factor released into the microenvironment (207), we decided that it may be more probable that iTregs were producing IL-10 as the soluble factor inhibiting the proliferation of effector T cells. To determine the extent to which iTregs were producing IL-10 we induced iTregs in culture and then treated with monensin, a GogliStop protein transport inhibitor, in the absence (Figure 3.9A) or presence (Figure 3.9B) of PMA and ionomycin for two hours. After two hours we analyzed the intracellular accumulation of IL-10 using flow cytometry. We found that iTregs in both the absence and presence of a strong activation with PMA and ionomycin were producing IL-10 (Figure 3.9), suggesting that IL-10 may be the soluble factor in iTregs contributing to the regulation of effector T cell proliferation. All of these data lead to us to our working model that the CD4⁺ T cells that differentiate into iTregs are highly proliferative cells that must undergo a substantial round of proliferation before developing into iTregs (Figure 3.10). Following proliferation, signaling network rewiring occurs that induces transformation of the T cells into iTregs (Figure 3.10).
Figure 3.9: iTreg IL-10 production
Purified human primary CD4+CD25− T cells are cultured for 5-6 days in iTreg polarizing media and then treated with monensin, a GogliStop protein transport inhibitor in the absence (A) or presence (B) of PMA and ionomycin for 2 hours. The treated T cells are then analyzed by flow cytometry using antibodies against CD25, CD45RA, FoxP3 and IL-10. The histogram represents increasing amounts of IL-10 expression in the different T cell populations (based on CD25, CD45RA and FoxP3 expression). iTregs produce the highest level of IL-10 with or without strong activation with PMA and ionomycin. Figure is representative of three independent donors.
 iTreg development may occur in two main steps. The CD4$^+$ T cells first undergo a significant round of proliferation in culture. Once divided, the cells then undergo a rewiring of signaling networks which leads to differentiation of the T cells into iTregs.
3.3 Summary

In summary, primary human CD4⁺ T cells will differentiate *ex vivo* into iTregs if cultured in the presence of anti-CD3, IL-2 and TGF-β. We are able to generate relatively large amounts of iTregs (20-25% iTregs in culture). iTregs start to develop around day three in culture and have fully developed after five to six days in culture from a population of highly proliferative T cells that have undergone significant rounds of proliferation before differentiating into iTregs. We have also been able to optimize our cell culture system to generate the maximum amount of iTregs possible. Human iTregs produced in this manner do appear to exhibit phenotypic markers of regulatory T cells, as they express high levels of CD25, FoxP3, CTLA4, GITR, along with some other markers that are typically present on Tregs (CD86, CCR2, CCR1, CD58, and CD30). iTregs failed to express CD45RA, CD127 and CD31. iTregs suppress the proliferation of target effector T cells even at a low ratio of 3 effectors to 1 iTreg through cell-to-cell contact along with soluble factors, like IL-10, which are probably involved. The data in this chapter suggest that our cell culture system may be able to generate a sufficient quantity of iTregs to use them as a therapeutic option in treatment of human disease. Also, the development of this novel cell culture system to induce human iTregs gives us a near ideal experimental platform to study the signaling molecular mechanisms involved in their development.
Chapter 4: Proximal TCR Signaling in Human iTregs

4.1 Introduction

The physiological relevance of Tregs in the control of the immune response emphasizes the importance of understanding the molecular mechanisms underlying their development and function. The intrinsic limitations of studying iTregs have prevented a comprehensive understanding of the differential signaling events that govern the development and function of Tregs. Sauer et al. described some of the signaling mechanisms in regulatory T cells when they found that the AKT/PI3K pathway inhibits FoxP3 expression in regulatory T cells (145). Moreover, constitutive activation of AKT in regulatory T cells leads to increased proliferation and concomitant abrogated suppressive capacity (147), further supporting the requirement for a “silent” AKT to sustain Treg function. We have taken advantage of our unique cell culture system outlined in Chapter 3 to obtain a better understanding of the signaling events that contribute to the altered proximal TCR-mediated activation of iTregs. We propose that differentiation from CD4+ T cells to iTregs involves the rewiring of the signaling network associated with TCR–dependent events upstream of the AKT pathway.

The TCR becomes activated in response to binding to a complementary MHC/peptide complex. Upon ligation, the TCR changes conformation allowing the ITAMs on CD3 to be exposed, and the Src-family kinase Lck to phosphorylate the ITAMs. Recent reports propose that a pool of pre-activated-Lck gains access to the ITAM sequences within the CD3/TCRζ complex exposed by the conformational changes
secondary to TCR engagement (208, 209). In this model, the critical priming step in the initiation of the TCR pathway would be the substrate accessibility (i.e. CD3 and TCRζ cytoplasmic tails) to the pool of available Lck, rather than Lck activation itself. Once Lck is recruited to the TCR complex, a subsequent kinase, Zap70, is recruited. Following recruitment, Zap70 will then be phosphorylated and activate the scaffolding protein LAT which will further recruit additional adaptor proteins, eventually leading to the activation of PI3K and generation of IP₃ among other second messengers. PI3K activation directly leads to AKT activation while IP₃ generation sparks a wave of signaling cascades that will induce Ca²⁺ influx into the T cell. Continued activation of the TCR signaling pathway through stimulation of the TCR is required for long-term T-cell activation and is dependent upon sustained Ca²⁺ influx. In order for Ca²⁺ influx and, thus, TCR signaling, to be sustained, an electrostatic imbalance between the intra/extracellular environments is maintained by K⁺ efflux through Kv1.3, KCa3.1 and other ion channels. The TCR signaling cascade is very rapid. Ligation with MHC/peptide complex leads to numerous downstream events inducing the activation, proliferation and differentiation of the T cell.

We hypothesize that the phenomenon of “silent” AKT is also occurring in human iTregs and may account for a defect in upstream events. In this chapter, we examine the events upstream of AKT activation following TCR stimulation. This data will lead to a better understanding on how AKT activity is blocked in iTregs shedding light on the possible molecular rewiring occurring during iTreg development.
4.2 Results

4.2.1 TCR-dependent signaling events in iTregs

To investigate the TCR signaling induced in human iTregs we cultured CD4\(^+\) T cells in iTreg polarizing conditions (anti-CD3, IL-2 and TGF-β) for 5-6 days, purified them using flow cytometry and analyzed the TCR signaling upon CD3 crosslinking. As expected, iTregs generated in our conditions acquired specific Treg signaling trademarks. Notably, iTregs exhibited a low activation of AKT in response to TCR engagement (Figure 4.1B). In contrast, remarkably effective ERK activation was evident (Figure 4.1B) along with substantially increased pattern of phosphotyrosine in several, but not in all, proteins as compared to effector T cells (Figure 4.1A), indicating that the blockage of the AKT pathway in iTregs did not occur due to a general failure of the TCR signaling machinery. iTreg cells exhibited the highest levels of active Lck in mock-treated samples under normal resting conditions (2 hours on ice), as indicated by the intense phosphorylation of Lck at Y394 (Figure 4.1B). However, iTregs displayed a reduced capacity to increase Lck activation in response to TCR ligation possibly because of high basal Lck activity in rested cells. This decreased ability to activate Lck was not derived from a difference in total protein or gene expression of Lck in iTregs compared to effector T cells (Figure 4.1C). These data suggest that human iTregs exhibit the same inhibition of the AKT pathway as seen in other Treg populations; however this doesn’t appear to be caused by a total failure of TCR signaling. Additionally, our data indicates that Lck is not further activated in response to TCR signaling but is active in rested iTregs.
Figure 4.1 A
Figure 4.1B

**Cell lysates**

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<td>-</td>
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**Ratio**

**Phosphorylated/Total Protein**

- CD3 X
- Y416Src
- Y159LAT
- S473AKT
- AKT
- ERK
- ERK1
- ERK2

**Bars**

- pSrc / Fyn-Lck
- pAKT / AKT
- pLAT / LAT
- pERK / ERK

Legend:
- Mock
- CD3x
Figure 4.1 C

Cell lysates

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Gene Expression

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</table>
**Figure 4.1: TCR-dependent signaling in iTregs.**

**(A)** Sorted CD4⁺CD25⁻, effector and iTregs were rested for 2 hours on ice, activated (+) or not activated (-) by anti-CD3 and anti-mouse IgG cross-linking for 3 min, then subsequently lysed (5 X 10⁵ cells) and analyzed by Western blotting for the presence of phosphotyrosines. The amount of phosphotyrosines was indicative of the level of TCR signaling activated in each cell type. **(B)** Sorted CD4⁺CD25⁻ T cells, effector T cells and iTregs were rested for 2 h on ice and activated (+) or not activated (-) by anti-CD3 crosslinking for 3 min. Lysates of 5 X 10⁵ cells were analyzed by Western blot to detect activated (phosphorylated) forms and total protein expression of Lck/Fyn (anti-pY416-Src recognizes both pY394-Lck and pY417-Fyn), AKT, LAT and ERK. GAPDH expression demonstrated equivalent loading. Protein band densities were quantified by densitometry. Ratio of phosphorylated:total protein was normalized to that of mock-treated CD25⁻ cells and depicted as mock-treated (empty bars) or CD3 crosslinked (black bars) cells. **(C)** Lysates from 5 X 10⁵ cells were analyzed by Western blot to detect the pattern of expression of proteins involved in early events of TCR signaling. Protein band densities were quantified and normalized to that of CD25⁻ cells as indicated below each blot. Transcription of the LCK gene was quantified from CD25⁻ T cells, effector T cells and iTregs. FoxP3 (as a positive control) and GAPDH (housekeeping gene) were assessed as controls. Briefly, total RNA was extracted from 5 to 7 X 10⁶ sorted cells. cDNA was obtained by reverse transcription and used as a template in a microarray analysis with the Affymetrix GeneChip System (Human Genome U133 Plus 2.0 Array microchip). The results represent the comparative mean value of triplicates from three different donors relative to CD25⁻ samples; bars represent the experimental range of the triplicates. The results were validated with real-time PCR (not shown). Reproduced with permission from (148).
4.2.2 Lck relocates to the highly active IL-2 receptor

One phenotypic attribute of iTregs is the high expression of CD25, the IL2-Rα subunit that associates with the IL2-Rβ (CD122) and the common γ (CD132) chains to form the high affinity IL2-R complex. In conjunction with the requirement for a constant supply of IL2, the enhanced expression of the high affinity IL2-R complex underscores the critical role that the IL2/IL2-R pathway plays in iTreg development (184, 210). In this context, the fact that Lck exhibited a similarly enhanced activity in iTregs when the TCR signaling machinery was not triggered suggests that an alternative pathway might activate Lck. In order to assess the potential involvement of the highly active IL2-R, we performed co-immunoprecipitation assays of Lck from effector T cells and iTreg lysates. Our results confirmed that the physical association between Lck and the IL2-Rβ subunit occurred only in iTregs (Figure 4.2A). In addition, Lck appears to be activated upon IL2-R engagement in iTregs. Sorted iTreg cells rested for an additional period of time (6-7 hours on ice) revealed the activation of TCR-dependent downstream events (as depicted in Figure 4.2B by the phosphorylation of LAT), though the actual level of activated Lck was essentially identical for TCR cross-linked and mock-activated cells. Conversely, a marked increase of Y416-phosphorylated Src was detected in iTreg cells treated with IL2 that paralleled the IL2-R-induced phosphorylation of STAT5. In agreement with others (211, 212), these findings support the direct involvement of Lck in the IL2-R signal transduction pathway of iTregs (Figure 4.3). As we observed no differences in Lck expression between conventional T cells and iTregs (Figure 4.1C), these results are
consistent with the redistribution of Lck to the IL2-R cluster occurring during the development of iTregs.
Figure 4.2

(A) Lysates from $10^7$ effector T cells and iTregs which were induced in our optimization media were incubated with Lck antibody-loaded agarose beads or Ig-isotype control (IgC). Cell extracts and immunoprecipitates were analyzed by SDS-PAGE and Western blots using CD122 (IL-2-β) and Lck antibodies. Quantification of co-immunoprecipitates was determined by the relative ratio of IL-2-β-bound to Lck and normalized to 1.0 in effector cells. (B) Sorted iTregs which were induced in our optimization media were rested for 6–7 h on ice and mock-activated, anti-CD3 crosslinked for 3 minutes or IL-2 treated for 5 hours. Lysates of 5 X $10^5$ cells were subjected to immunoblotting using the indicated antibodies. LAT expression verified equal loading. Quantification of protein expression was normalized on the basis of mock-treated iTregs for each individual blot. Representative of three independent donors. Reproduced with permission from (148).
Figure 4.3: LCK relocates to the IL-2R in iTregs. Upon TCR engagement with MHC bound to peptide will lead to a series of signaling events conveying activation of the Src-family linked kinase Lck and Zap70 in effector T cells. Once these kinases are activated, subsequent downstream adaptor proteins are phosphorylated leading to the generation of second messengers which induce T cell differentiation, activation and proliferation. We have found that Lck relocates to the highly activated IL-2R complex in iTregs. Upon IL-2 binding to IL-2R, Lck is phosphorylated/activated, suggesting that Lck is rewired from the TCR to the IL-2R and is involved in IL-2 signaling.
4.2.3 Impaired LAT activation in iTregs

Because we have demonstrated a partial defect in the TCR signaling pathway of iTregs in proteins like Lck and AKT, we wanted to investigate additional members of the TCR pathway for changes in signaling profile. LAT is a scaffolding protein phosphorylated in response to TCR stimulation which couples antigen receptor activation to downstream signaling cascades (213) making it an attractive candidate for differential regulation in iTreg signaling. We set out to examine where or not LAT became differently phosphorylated after TCR stimulation in Tregs compared to effector T cells. Purified human CD4⁺ T cells were cultured in iTreg polarizing media (anti-CD3, IL-2 and TGF-β) for 5-6 days. Sorted iTreg and effector T cell populations were rested for 6-7 hours on ice and then activated with anti-CD3 for TCR signaling or IL-2 for IL-2R signaling. STAT5 was phosphorylated in iTregs and effector T cells suggesting that IL-2R signaling was activated. In agreement with what has been previously demonstrated by others (145), AKT was activated in effector T cells but was inactive in iTregs in response to CD3 crosslinking (Figure 4.4). LAT phosphorylation was partially impaired in iTregs when compared to effector T cells (Figure 4.4), implying that relocation of Lck to the IL-2R could be affecting downstream signaling and activation of LAT. PLC-γ is recruited to the LAT complex upon TCR stimulation and relays information from the activated receptor to downstream signaling cascades through the production of second messengers which initiate the Ca²⁺ influx in T cells (214). We found that PLC-γ phosphorylation was substantially lower in iTregs compared to effector T cells (Figure 4.4), suggesting that downstream events such as Ca²⁺ flux may also be effected.
Figure 4.4: LAT phosphorylation is impaired in iTregs
Sorted effector and iTregs were rested for 6-7 hours on ice and mock-activated, anti-CD3 crosslinked for 3 minutes or IL-2 treated for 5 hours. Lysates of 5 X 10^5 cells were subjected to immunoblotting using the indicated antibodies. The western blot was probed for the expression of P-STAT5, STAT5, P-AKT S473, P-LAT Y191, LAT, P-Zap70 Y493, Zap70, P-PLCγ Y793 and GAPDH expression was used to verify equal loading. Representative of three independent donors.
4.2.4 Functional dissociation between Kv1.3 and TCR activation in iTregs.

It has been previously demonstrated that, in contrast to conventional CD4+ and CD8+ T cells, the activation of the K+ channel Kv1.3 is refractory to TCR stimulation in human nTregs (189). To explore whether iTregs displayed the same functional trait along with the acquisition of other regulatory/suppressor trademarks, we analyzed the response of Kv1.3 to TCR engagement among different T cell subsets. We first examined the protein and gene expression levels of Kv1.3 in iTregs compared to effector T cells and found that there was no significant difference between Kv1.3 protein and gene expression between the populations (Figure 4.5). We next investigated whether Kv1.3 was activated in response to TCR stimulation in iTregs. The Ca^{2+} flux was evaluated in iTregs upon TCR stimulation in the presence of or absence of the Kv1.3 inhibitor margatoxin. We determined that Ca^{2+} flux in response to TCR stimulation was inhibited in naïve T cells upon inhibition of the Kv1.3 channel, however iTreg Ca^{2+} flux remained relatively unaffected (Figure 4.6), indicating that TCR induced Ca^{2+} flux was not dependent on Kv1.3 activity in human iTregs.

To directly confirm that Kv1.3 was not activated by the TCR in iTregs we took advantage of an automated, high-throughput patch-clamp screening assay. High-throughput patch-clamp assay is a revolutionary method to measure activity of ion channels within an entire population of cells (0-384 cells) simultaneously. Unlike conventional patch clamp techniques where ion channel activity is measured in one cell at a time, this method allowed us to visualize the complete distribution of ion channel activity within the entire population of cells at once. To perform this highly specialized
technique, cells were plated in a 384 well plate and the depolarization potential was read across the cell membrane in the presence or absence of Kv1.3 inhibitor. Figure 4.7A depicts a representative spread of Kv1.3 activity obtained in human effector T cells. The advantage of this method for measuring Kv1.3 activity is demonstrated in Figure 4.7A where a widespread distribution of Kv1.3 activity can be visualized within a single population. It is much more difficult to obtain such a complete cross-sectional picture of channel activity using traditional patch clamping techniques. Concise graphical representation of this distribution is illustrated in Figure 4.7B. Using this system to measure Kv1.3 activity in our T cell populations, we found that effector T cells had a substantial increase in Kv1.3 activity upon TCR stimulation when compared to CD25− T cells, indicating that Kv1.3 is active at the TCR and may be regulating the TCR induced Ca²⁺ flux in effector T cells (Figure 4.8). However we identified a similar profile of weak Kv1.3 response upon TCR stimulation in both induced and natural Tregs (Figure 4.8). Combined with the observation of steady Kv1.3 mRNA and protein expression (Figure 4.5), these findings suggest the functional dissociation between TCR and Kv1.3 activity is likely due to the remodeling of the TCR signaling network during iTreg differentiation.
Figure 4.5: Kv1.3 expression in iTregs
Sorted CD4⁺CD25⁻ T cells, effector T cells, and iTregs were analyzed by Western blot to compare the expression of Kv1.3. The double-banded pattern depicted in effector and iTreg Kv1.3 blots likely correspond to different glycosylation forms of Kv1.3 (215). Quantification of protein expression was normalized to the band density of CD25⁻ cells. GAPDH levels demonstrate equal protein loading. Gene expression was determined as detailed in Figure 4.1C. Reproduced with permission from (148).
Figure 4.6: TCR-induced Ca\(^{2+}\) flux is not affected in iTregs upon inhibiting Kv1.3

Primary human CD\(^{4+}\) T cells were cultured for 5-6 days in iTreg polarizing media (anti-CD3, IL-2 and TGF-β). Cells were then stained with Indo-1 AM calcium dye and incubated for 30 min at 37°C. Indo-1 treated cells were washed and further treated with 60 nM Margatoxin, a Kv1.3 inhibitor, for 15 min at 37°C. The cells were then placed on ice and labeled with biotinylated anti-CD3, CD25 and CD45RA. Baseline readings were taken for 30 seconds and the avidin (5 mg/mL) was added to induce CD3 crosslinking. The samples were then read for 5 minutes. T cell populations were distinguished by their expression of CD25 and CD45RA: naïve cells by their expression of CD45RA\(^+\)CD25\(^-\) and iTregs cells by their expression of CD45RA\(^-\)CD25\(^{Hi}\). The Ca\(^{2+}\) flux shown comes from extracellular Ca\(^{2+}\) added to the samples. To determine percentage inhibition, Ca\(^{2+}\) flux peak values were calculated for each population in RPMI medium only and set equal to 100 percent. Ca\(^{2+}\) flux peak values in the presence of Margatoxin were subtracted from Ca\(^{2+}\) flux peak values in the presence of RPMI only. This difference is the percentage inhibition.
Figure 4.7: High-throughput method for measuring Kv1.3 ion channel activity
(A) Represented picture of Kv1.3 current obtained from 63 human primary effector T cells. The intensity of Kv1.3 activity varies from cell to cell, therefore measuring Kv1.3 activity in a whole population of cells is critical to obtain a representative activity of the whole population. (B) Representative graph of Kv1.3 activity in a population of effector T cells. Whiskers represent the 10th and 90th percentiles of experimental values; boxes are limited by the 25th and the 75th percentiles; the solid line is the median and the dark square represents the mean value.
Figure 4.8

Figure 4.8: Kv1.3 activity in iTregs
CD4^+CD25^- T cells and nTregs isolated from the same donor were cultured in iTreg polarizing medium (anti-CD3, IL-2 and TGF-β). After 6 days, CD4^+CD25^- T cells, effector T cells and iTregs were sorted and maintained for 60 h in serum-free AIMV medium with or without 0.15 mg/mL of soluble anti-CD3 antibody. Kv1.3 current in sorted cells and nTregs was measured with high-throughput patch-clamping screening. Experimental values represent the combined distribution of Kv1.3 measurements on individual cells isolated from three independent experiments. N = 255 CD4^+CD25^- cells; N = 186 effector cells; N = 88 nTregs; N = 129 iTregs. The distribution of Kv1.3 values is depicted in box plots with whiskers representing the 10th and 90th percentiles of experimental values; boxes are limited by the 25th and the 75th percentiles; solid lines are the medians, and dark squares represent the mean values. A two-sample Kolmogorov–Smirnov test was used to compare paired distributions of Kv1.3 currents. ** represent p<0.01. Reproduced with permission from (148).
4.2.5 Regulation of Kv1.3 function by the IL2-R pathway in iTregs.

During the formation of the immune synapse, association between the Src-family tyrosine kinase Lck and Kv1.3 is necessary for the initiation of the TCR-mediated signaling (216-218), and has been reported in other, non-TCR-dependent T cell responses as well (219-221). Therefore, we explored whether a differential Lck activation in iTregs may occur simultaneously with the loss of control of Kv1.3 activity by the TCR. The functional incidence of Kv1.3 spatial redistribution has been documented in T cells (216-221) and association of Kv1.3 with novel signaling partners and redistribution of Kv1.3 to areas of increased signaling activity has recently been demonstrated. In 2000, Levite et al. reported a physical and functional association of Kv1.3 with beta1 integrin in T cells, suggesting that integrin activation may be regulated by Kv1.3 (220). Additionally, it was shown that Kv1.3 localized with CD3 in T cells and appeared to be translocating to the immunological synapse formed between cytotoxic and target cells (216, 217) suggesting a physical relocation of Kv1.3 to areas of increased signaling activity. Kv1.3 channel compartmentalization in the immunological synapse has also been identified in systemic lupus erythematosus (218) suggests that Kv1.3 distribution can differ in pathologic states. To examine whether TCR-dissociated Kv1.3 becomes functionally linked to the highly active IL2-R complex, we performed high-throughput patch clamp analysis with sorted iTregs incubated with IL-2, IL-6 or IL-10. In contrast to the weak response observed upon TCR ligation (Figure 4.8), iTregs in culture with IL-2 sustain significantly larger magnitude of Kv1.3 currents compared to cells cultured with IL-6 or IL-10 (Figure 4.9). These results provide, to our knowledge, the first evidence of the
functional integration of Kv1.3 into the IL2-R pathway in human T cells. Moreover, the
linkage of Kv1.3 to the highly Lck-activating IL2-R coincides with the functional uncoupling from TCR control (Figure 4.10).
Sorted iTregs were cultured for 60 h in AIMV with or without 5 ng/mL of IL-2, IL-6 or IL-10. Depiction and measurement of Kv1.3 currents were performed as described in Figure 4.9. In iTregs Kv1.3 was found to be response to IL-2 stimulation. Profiles of Kv1.3 currents represent the combined values of two independent experiments. A two-sample Kolmogorov–Smirnov test was used to compare paired distributions of Kv1.3 currents. ** represent p<0.01. Reproduced with permission from (148).
Figure 4.10: Kv1.3 relocates to the IL-2R

TCR engagement with MHC bound to peptide leads to a series of signaling events conveying activation of the Src-family linked kinase Lck and Zap70 in effector T cells. Once these kinases are activated, subsequent downstream adaptor proteins are phosphorylated leading to the generation of second messengers which activate the Kv1.3 ion channel and induce T cell differentiation, activation and proliferation. In iTregs, Lck and Kv1.3 relocate to the highly activated IL-2R complex, which, upon IL-2 binding to IL-2R Lck, is phosphorylated/activated along with Kv1.3 suggesting that Lck and Kv1.3 are functionally linked and are relocated from the TCR to the IL-2R for their involvement in IL-2 signaling.
4.2.6 $\text{Ca}^{2+}$ flux in response to TCR stimulation remains intact in iTregs

Given our demonstration of a partial loss of TCR signaling in iTregs and relocation of Lck and Kv1.3 from the TCR to the highly active IL-2R, relocation of functionally linked Lck and Kv1.3 to the active IL-2R could have downstream effects on T cell $\text{Ca}^{2+}$ influx. Therefore, we next investigated the status of TCR induced $\text{Ca}^{2+}$ flux in iTregs. Using the Indo-1 dye to assess $\text{Ca}^{2+}$ concentration, we demonstrated higher intracellular concentrations of $\text{Ca}^{2+}$ in iTregs than effector T cells upon TCR stimulation (Figure 4.11). As Kv1.3 was unavailable to contribute to the electrochemical gradient normally permissive to $\text{Ca}^{2+}$ influx, this data implicated an additional $\text{K}^+$ ion channel linked to the TCR in iTregs.
Figure 4.11: TCR-induced Ca$^{2+}$ flux remains intact
T cell populations were distinguished by expression of CD25 and CD45RA: effector T cells in orange, iTregs in green, memory in blue and naïve in red. The Ca$^{2+}$ flux shown is from extracellular Ca$^{2+}$ added to the samples. The iCa$^{2+}$ index was normalized to the baseline reading before TCR signaling was induced for each population.
4.2.7 Regulatory switch of Kv1.3 and KCa3.1 in iTreg TCR signaling

Due to the fact that Ca^{2+} flux remains intact in iTregs, we explored the possibility that in iTregs another K^+ ion channel was compensating for the lack of Kv1.3 at the TCR. A likely candidate for this role in iTregs, KCa3.1, is a K^+ ion channel expressed on T cells that, like Kv1.3, is also activated in response to increases in intracellular Ca^{2+}. We first examined the expression level of KCa3.1 and identified a significant increase in both KCa3.1 gene and protein expression in iTregs compared to effector and CD25^- cells (Figure 4.12). Subsequent evaluation of KCa3.1 activity in response to TCR stimulation found that TCR-induced Ca^{2+} flux was substantially reduced compared to that of naïve cells upon treatment with a KCa3.1 inhibitor, TRAM34 (Figure 4.13). These data suggest a regulatory switch of K^+ channel activity in iTregs that may constitute a key contributing factor in the signaling rewiring associated with the development of peripheral human iTregs and sheds new light upon the reciprocal cross-talk between TCR and IL2-R pathways. Confirmation of this finding by high-throughput patch clamp experiments is ongoing.
Figure 4.12: KCa3.1 expression in iTregs
Sorted CD4⁺CD25⁻ T cells, effector T cells, and iTregs 5 X 10⁵ cells were analyzed by Western blot and microarray to compare the expression of Kv1.3. Quantification of protein expression was normalized to the band density of CD25⁻ cells. GAPDH levels demonstrate equal protein loading. Gene expression was determined as detailed in Figure 4.1C.
Figure 4.13: TCR-induced Ca$^{2+}$ flux is inhibited in iTregs by suppressing KCa3.1 activity

Primary human CD4$^+$ T cells were obtained and cultured for 5-6 days in iTreg polarizing media (anti-CD3, IL-2 and TGF-β). We then stained the cells with Indo-1 AM calcium dye and incubated them from 30 min at 37°C. Indo-1 treated cells were washed and further treated with 60 nM TRAM34, a KCa3.1 inhibitor, for 15 min at 37°C. The cells were then placed on ice and labeled with biotinylated anti-CD3, CD25 and CD45RA. Baseline readings were taken for 30 seconds and then avidin (5 mg/mL) was added to induce CD3 crosslinking. Samples were then read for 5 minutes. The T cell populations were distinguished by expression of CD25 and CD45RA: naïve cells by their expression of CD45RA$^{+}$CD25$^-$ and iTregs cells by their expression of CD45RA$^{+}$CD25$^{hi}$. The Ca$^{2+}$ flux shown is from extracellular Ca$^{2+}$ added to the samples. To determine percentage inhibition, Ca$^{2+}$ flux peak values were calculated for each population in DMF medium only and set equal to 100 percent. Ca$^{2+}$ flux peak values in the presence of TRAM34 were subtracted from Ca$^{2+}$ flux peak values in the presence of RPMI only. This difference is the percentage inhibition.
4.3 Summary

In contrast to effector T cells in which robust activation of the TCR signaling pathway occurs, human iTregs undergo partial activation of the TCR, as a consequence of a rewiring in the TCR signaling pathway. We have shown that iTregs have a blockage in the AKT pathway in response to the TCR consistent to what has previously been found by others in mouse and human Tregs. Additionally, we have identified higher Lck activity in rested iTregs compared to effector T cells. However, upon TCR stimulation, Lck activation is not increased above resting levels in iTregs which could be caused by Lck redistribution from TCR to the IL-2R. Haughn et al. (222) demonstrated that a very active, high-affinity IL2-R in T cells might cause the functional uncoupling of the TCR signaling machinery through diversion of the subcellular localization of Lck to the IL2-R multiprotein. Consistent with this model, strong activation of Lck by the IL2-R pathway in iTregs may compromise the amount of Lck available in CD3/TCR complexes thereby altering the TCR signaling cascade. In agreement with other reports (216-218), our findings also suggest that Kv1.3 remains functionally linked with the pool of Lck susceptible to lateral mobility, providing a mechanism for redistribution of Kv1.3 in iTregs. Overall, our results are consistent with a model by which iTreg development requires a two-step process whereby cells undergo a significant round of proliferation followed by a rewiring of the TCR signaling inducing iTreg development. Interestingly, differentiation into iTregs occurs preferentially in cells that undergo strong proliferative expansion, which involves continuous TCR-mediated regulation of Kv1.3 function along with strong activation of the AKT pathway. During transition to the differentiation stage and subsequent acquisition of the suppressor phenotype, the formation of new protein
complexes occurs concurrently with the remodeling of functional signaling paths that eventually dissociate AKT and Kv1.3 activities from the TCR network. We provide evidence for uncoupling of early TCR signaling elements, in addition to other potential mechanisms (173), through the competitive cross-talk between TCR and IL2-R pathways in cells with high IL2-dependent activity. These findings support a dynamic model by which the redistribution of common, key signaling components (Lck-Kv1.3) may represent a rapid and efficient mechanism of adapting the cell signaling machinery to a new functional context. The functional switch from antigen-dependent signaling in effector cells to cytokine-dependent responsiveness in iTregs is consistent with the physiological prevalence of the suppressor activity of iTregs upon antigen clearance (148).

We also identified in this chapter that despite the fact that Kv1.3 relocates to the IL-2R, TCR induced Ca\textsuperscript{2+} flux remains intact, indicating that there is another ion channel compensating for its relocation. We have data to suggest a regulatory switch of K+ channel activity in iTregs from Kv1.3-TCR to KCa3.1-TCR. This data is of particular importance clinically because of an ongoing clinical trial targeting Kv1.3 activity on T\textsubscript{EM} cells in MS patients to inhibit the harmful effects of the effector T cells in the onset and progression of this disease. However, special attention should be paid to the effects of inhibiting Kv1.3 on iTregs, which may affect IL-2R signaling, possibly decreasing regulatory T cells function thus exacerbating the disease.

Collectively, these results: (1) provide a novel mechanistic link between the remodeling of a signaling network and the acquisition of suppressor iTreg phenotype
with the selective relocation of TCR-associated proximal components; (2) support the importance of the finely-tuned TCR/IL2-R cross-talk in the control of T cell fate decisions, and (3) underscore the differential role that $K^+$ channels may play in specific T cell subpopulations and/or their functional responses.
Chapter 5: Blockade of AKT Activity and the Association of AKT with SMAD3

5.1 Introduction

One key distinguishing feature of Tregs is that the PI3K/AKT pathway antagonizes their development and function (145, 146). Constitutive activation of AKT prevents TGF-β induced FoxP3 expression in T cells (145, 146) significantly impairing Treg development. Additionally, constitutive activation of AKT in nTregs leads to increased proliferation and concomitant abrogated suppressive capacity (147) suggesting that “silent” AKT is crucial for Treg development and function. Our lab has investigated the blockade of the AKT pathway in human iTregs and has shown that, like in mouse and human nTregs, the TCR-induced AKT pathway is severely inhibited (148). We further examined the early signaling events in the TCR signaling pathway and found a functional re-organization of the protein complex involved in TCR signaling in human iTregs.

We next investigated the functional connection between re-wiring of the TCR signaling pathway and the lack of AKT activation occurring in iTregs. Ligation of the TCR with MHC/peptide complex results in a series of signaling cascades that activates PI3K, leading to the phosphorylation of PtdIns (4,5)P₂ and, with lower efficiency, PtdIns 4P, at the 3'-OH position of the inositol ring to produce PtdIns (3,4,5)P₃ and PtdIns (3,4)P₂, respectively. The production of PtdIns (3,4,5)P₃ will recruit AKT to the cell membrane through binding by AKT’s PH domain. After translocating to the membrane, AKT is then phosphorylated by PDK1 and/or mTOR leading to the phosphorylation of both S473 and T308 activating AKT. Once activated, AKT will phosphorylate several
downstream targets leading to T cell activation, proliferation and differentiation. AKT activity is negatively regulated by several phosphatases. PTEN and SHIP1 de-phosphorylate PtdIns \((3,4,5)P_3\), reducing the amount of active AKT at the cell membrane. Additionally, PP2A and PHLPP are phosphatases that directly de-phosphorylate AKT, causing it to become inactivated.

iTreg differentiation requires blockage of the TCR AKT pathway as well as concurrent induction of TGF-\(\beta\) and IL-2 signaling pathways. TGF-\(\beta\) is a cytokine produced by lymphocytes, macrophages and dendritic cells, that affect the differentiation, proliferation and activation of immune cells (176, 177). Signaling through the TGF-\(\beta\) pathway will inhibit T and B cell proliferation and induce apoptosis as well as prevent \(T_{H1}\), \(T_{H2}\), and CTL differentiation. TGF-\(\beta\) signaling is initiated by ligation of extracellular TGF-\(\beta\) with TGF-\(\beta\)RII which will, in turn, trans-activate TGF-\(\beta\)RI. Once activated TGF-\(\beta\)RI phosphorylates intracellular SMAD3 enabling it to change confirmation thereby permitting the assembly of homo- and hetero-oligomeric complexes with itself or SMAD4, respectively. These SMAD3 complexes express a nuclear location sequence facilitating their translocation into the nucleus where they regulate transcription through direct DNA binding. SMAD3 complex formation enables its interaction with additional DNA-binding cofactors in order to achieve high affinity binding to specific target genes (223). One such target gene in Tregs is the enhancer of FoxP3, suggesting that TGF-\(\beta\) signaling induces FoxP3 expression during Treg development (186, 187). Additionally, SMAD3 associates with a FoxP3 promoter where it forms part of a complex that induces FoxP3 expression (224). Intriguingly, it has been reported that
induction of FoxP3 enhancer activity by SMAD3 is required for iTreg development providing further support for this interaction (175).

In this chapter, we set out to identify the distal TCR signaling events that govern AKT blockage in iTregs. We report a differential regulation of kinases to phosphatase ratio in iTregs compared to conventional T cells. Moreover, we report a new, non-enzymatic function for AKT in iTregs and its cross-talk with the TGF-β pathway. These data have led to a better understanding of how iTregs develop and also revealed a novel target for potential modulation of iTreg differentiation.
5.2 Results

5.2.1 Blockage of AKT activity in iTregs

We found that protein expression of AKT was significantly higher in iTregs when compared to naïve and effector T cells by Western blot and flow cytometry (Figure 5.1A-C) without a concomitant increase in AKT gene expression (Figure 5.1D), implying that AKT expression is regulated post-transcriptionally. Unlike in naïve and memory T cells, AKT phosphorylation at S473 in iTregs does not increase due to CD3 crosslinking (Figure 5.2A, B), therefore human iTregs also have a blockage of TCR induced AKT activation. P-AKT T308 levels followed the same pattern of phosphorylation as P-AKT S473 (Figure 5.2C). To examine whether the lack of AKT activation was due to a loss of the cells’ general ability to phosphorylate AKT or if it was due to a TCR-specific anomaly, iTregs were activated with pervanadate, a phosphatase inhibitor that leads to non-specific phosphorylation. Pervanadate treatment resulted in an increase in P-AKT S473 levels in iTregs (Figure 5.2A, B) indicative of a TCR-specific AKT activity blockage. Previously we have shown that stimulation of TCR activates the ERK pathway in iTregs (Figure 4.1B), suggesting this blockage is not due to a complete loss of TCR signaling but is AKT specific (148). These results indicate a TCR-induced blockage of AKT activity in human iTregs and suggest that increased AKT protein expression is due to enhanced stability of AKT (225) resulting in subsequent AKT accumulation in the cytosol of Tregs.
Figure 5.1: Protein kinase AKT expression in iTregs

(A) Purified human T cells were obtained and cultured for 5-6 days in iTreg polarizing conditions. Lysates from 3 x 10^5 sorted CD25^+, CD25^low or iTreg cells were analyzed by Western blot to examine protein expression patterns. GAPDH was used as a loading control. (B) Total protein expression was analyzed using flow cytometry for AKT in various T cell subsets. (C) Quantification of AKT MFI from human donors using flow cytometry. (D) Regulation of gene expression. GAPDH (housekeeping gene) and FoxP3 (positive control) were assessed as controls. Data are representative of 3 independent donors.
Figure 5.2: TCR-induced blockage of AKT activity in iTregs

(A) Cultured human T cells were rested on ice for 5-6 hours and then stimulated with CD3 or pervanadate for 3 minutes. Flow cytometry was performed to detect phospho-AKT S473, CD25, CD45RA, FoxP3 and CTLA4. (B) Quantification of data in A was analyzed using the CD3 activation index of P-AKT S473 in T cells measured by flow cytometry. The P-AKT S473 index was determined by taking the percentage of MFI of P-AKT S473 upon CD3 activation and dividing it by MFI P-AKT S473 following pervanadate treatment (representing the max potential of P-AKT S473). Data were analyzed for statistical significant using repeated measures ANOVA with a Student Newman-Keuls multiple comparisons post-hoc analysis. (C) The P-AKT T308 index was determined by taking the percentage of MFI of P-AKT T308 upon CD3 activation and dividing it by MFI P-AKT T308 following pervanadate treatment (representing the max potential of P-AKT T308). Data were analyzed for significance using repeated measures ANOVA with a Student Newman-Keuls multiple comparison post-hoc analysis. ***p<0.001. **p<0.01. *p<0.05. These data are representative of 4 independent human donors.
5.2.2 Blockage of AKT activity in iTregs is not due to an inability to translocate AKT to the membrane

AKT activation requires its translocation to the membrane followed by sequential phosphorylation of AKT at S473 and T308 (74, 77, 78). Examination of AKT localization within T cells activated through crosslinking with anti-CD3 (Figure 5.3A) revealed areas of AKT-CD3 co-localization which were considered to be membrane bound AKT. Quantification of bound-AKT to total AKT was reported as a percentage of AKT recruited to the membrane (Figure 5.3B). Upon CD3 stimulation, significantly higher increase in AKT translocation to the membrane was observed in iTregs as compared to conventional T cells (Figure 5.3B). However conventional T cells did exhibit a higher percentage of AKT bound to the membrane in rested cells which is most likely due to strong PHA induced activation in the first culture (Figure 5.3B). The cell membrane contains micro domains of specialized lipid composition known as lipid rafts that allow localized signaling events to occur within discrete regions of the cell membrane. Lipid rafts function by compartmentalizing cellular processes, by serving as organizing centers for the assembly of signaling molecules, and by influencing membrane fluidity and protein trafficking (226, 227). To explore the membrane localization of AKT, we analyzed the composition of lipid rafts in iTregs compared to conventional T cells (Figure 5.4). We found substantially more AKT localization in lipid rafts in iTregs compared to conventional T cells (Figure 5.4), suggesting that AKT is translocating to areas of the membrane involved in active signaling. Additionally, Galectin-3 (Gal3), which is known to inhibit the formation of large lipid macro domains from smaller micro domains and is thought to limit TCR clustering necessary for TCR-mediated signal
initiation (228), is present at high levels in the lipid rafts of iTregs but was virtually undetectable in the membranes of conventional T cells. These results suggest that the localization of Gal3 to lipid rafts in iTregs could represent a component of the cellular mechanism of AKT blockage. Fyn and VCP were used as positive controls for the separation of lipid raft verses soluble fractions (Figure 5.4). Together these findings indicate that the blockage of AKT activity in iTregs is not because of its inability to translocate to the cell membrane and imply that the defective AKT signaling could be due, at least in part, to the inability to form functional protein complexes downstream of the TCR critically important for T cell activation.
Figure 5.3

A

<table>
<thead>
<tr>
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<tr>
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<td>DAPI</td>
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B

% of AKT Colocalization

- Conv. Mock
- Conv. CD3
- iTreg Mock
- iTreg CD3

Significance levels: *P < 0.05, **P < 0.01, ***P < 0.001
Figure 5.3: AKT is recruited to the membrane after TCR stimulation

(A) Conventional CD4⁺ T cells cultured with PHA and CD4⁺ T cells in iTreg optimization polarizing conditions (anti-CD3, IL-2, TGF-β) were obtained and rested for 7 hours on ice. Once rested, the cells were stimulated with (CD3) or without (Mock) CD3 antibody for 3 minutes. The cells were then treated with antibody against total AKT protein (green) and treated with DAPI stain for nucleus (blue). Deconvolution microscopy was performed to visualize the localization of AKT. This data is representative of three independent donors. (B) Quantification of AKT-CD3 colocalization. For quantification of images, the colocalization highlighter plugin from ImageJ was used to determine the colocalization of AKT-CD3 in 100 cells for each population. AKT-CD3 colocalization was considered to be membrane bound AKT. Membrane-bound AKT was expressed as a percentage of total AKT from each cell. Data were analyzed for significance using repeated measures ANOVA followed by a Student Newman-Keuls multiple comparisons post-hoc analysis. ***p<0.001. **p<0.01. *p<0.05. Error bars represent the SEM.


**Figure 5.4**

<table>
<thead>
<tr>
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**Figure 5.4: Lipid Raft components in iTregs**
Purified CD4<sup>+</sup> T cells were subjected to ultracentrifugation on a 5-80% sucrose gradient. Layers were analyzed by Western Blot analysis.
5.2.3 iTregs express different levels of phosphatase compared to conventional T cells

To determine whether the lipid composition on the cell membrane of iTregs was affecting AKT’s ability to translocate to the membrane, we assessed the expression of PtdIns phosphatases that are responsible for de-phosphorylating PtdIns (3,4,5)P$_3$ into an inositol derivative with lower affinity for AKT. PTEN de-phosphorylates the 3’-OH position of PtdIns (3,4,5)P$_3$ generating PtdIns (4,5)P$_2$ (81). iTregs express significantly higher levels of PTEN than memory or naïve T cells from the same culture (Figure 5.5). Overexpression of PTEN also correlated with an increase of P-PTEN in iTregs (Figure 5.5B, D), which could confer an increase in protein stability (229, 230). Additionally, we did not find any significant differences in the gene expression of PTEN between any T cell types (Figure 5.5A). The ratio of P-PTEN/PTEN was calculated by quantifying the mean fluorescence intensity (MFI) of P-PTEN and PTEN respectively using flow cytometry (Figure 5.5E). iTregs have a significantly higher P-PTEN/PTEN ratio than naïve cells, implying that the differences in PTEN expression are due to an increase of PTEN stability. These results suggest that the overexpression of PTEN may be a key event in signaling leading to the blockage of AKT and subsequent development of iTregs.

To investigate if PTEN activity is needed in the development of iTregs, we took advantage of the PTEN inhibitor VO-OHpic. VO-OHpic efficiency was validated in conventional T cells with mock, CD3 or CD3/CD28 crosslinking. Upon inhibition of PTEN in unstimulated or anti-CD3 conventional T cells there was no substantial difference in P-AKT S473 or P-Src levels compared to no inhibitor with CD3 activation alone. However, upon CD28 co-stimulation in the presence of PTEN inhibitor, increased
P-AKT S473 and P-Src were observed (Figure 5.6 A, B). These data suggest PTEN involvement in CD3/CD28 TCR signaling rather than in CD3 stimulation alone. The addition of the PTEN inhibitor to iTreg polarizing culture at various time points and concentrations did not affect the development of human iTregs (Figure 5.7), indicating that, although PTEN is overexpressed in iTregs, it does not appear to be a crucial signaling molecule involved in CD3 induced iTreg development.

We also examined the expression of SHIP1, the phosphatase that dephosphorylates the 5’OH position of PtdIns (3,4,5)P_3, generating PtdIns (3,4)P_2 (83). SHIP1 gene expression was increased in iTregs (Figure 5.8A), however SHIP1 protein expression in iTregs was not increased compared to other T cell populations (Figure 5.8B and C). These results suggest that SHIP1 is not involved in the TCR blockage of AKT activity seen in iTregs.
Figure 5.5

A

PTEN

1.00 1.10 1.46

1.00 1.00 2.08

FoxP3

1.00 7.20

59.0

CD25⁺

CD25⁻

CD25⁺

CD25⁻

GAPDH

1.00 0.98 0.88

1.00 1.60

1.00 2.08

mTOR

1.00

B

PTEN

Naive 1.0 1.0 1.4

Memory 1.0 1.0 1.2

Effector 3.5 6.2 1.7

iTreg

1.3 1.0 1.3 1.5

C

PTEN / GAPDH Ratio

Naive Memory Effector iTreg

* *

D

PTEN

Naive Memory iTreg

@-PTEN

Naive Memory iTreg

MFI

Naive Memory iTreg

E

©PTEN/PTEN Ratio

Naive Memory iTreg

*
Figure 5.5: PTEN is overexpressed in iTregs

(A) High levels of PTEN and mTOR gene expression in iTregs compared to other T cell populations were visualized using micro-array data. GAPDH (housekeeping gene) and FoxP3 (positive control) were assessed as controls. (B) Purified iTregs selectively express high levels of PTEN protein. Lysates from $3 \times 10^5$ sorted naïve, memory, effector or iTreg cells were analyzed by Western blot to detect the pattern of select protein expression. (C) Protein band densities were quantified by densitometry using Western blots shown in B. Ratios of total PTEN or phosphorylated PTEN:GAPDH are shown. Data were analyzed for significance using a repeated measures ANOVA followed by a Student Newman-Keuls multiple comparisons post-hoc analysis. Data are representative of 3-5 independent donors. (D) PTEN and Phospho-PTEN expression was assessed by flow cytometry in naïve, memory, and iTreg subsets. Quantification of PTEN and P-PTEN mean fluorescence intensity (MFI) from human donors. Data were analyzed for significance using a repeated measures ANOVA followed by a Student Newman-Keuls multiple comparisons post-hoc analysis. Data are representative of 4 independent donors. (E) Quantification of P-PTEN to PTEN ratio obtained from the MFI. Data were analyzed for significance using a repeated measures ANOVA followed by a Student Newman-Keuls multiple comparisons post-hoc analysis. ***p<0.001. **p<0.01. *p<0.05.
Figure 5.6

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- **GAPDH**

- **LAT**

- **LCK**

- **Src**

- **AKT**

- **mTOR**

- **-mTOR S2481**
Figure 5.6: Inhibiting PTEN in iTregs increases TCR activation only with CD3/CD28 stimulation

(A) Purified human CD4⁺ T cells were cultured in PHA. After 5-6 days in culture, the cells were washed, rested on ice for 5-6 hours and then subsequently treated with either 1 μM VO-OHpic (PTEN inhibitor) or 1 nM okadiac acid (PP2A inhibitor) for 30 minutes at 37°C in the presence of 5% CO₂. The cells were then mock-activated, CD3 activated or CD3/CD28 activated for 3 minutes. Lysates from 3 x 10⁵ cells were analyzed by Western blot to detect the pattern of select protein expression. Data are representative of 3 independent donors. (B) Protein band densities were quantified by densitometry from Western blots in A. The ratio of phosphorylated protein to total protein was normalized to that of mock-treated, no inhibitor cells and depicted as mock-treated (empty bars), CD3 crosslinked (black bars) cells or CD3/CD28 crosslinked (grey bars).
Figure 5.7

A

B
Figure 5.7: Inhibiting PTEN does not affect iTreg development

(A) Purified human CD4+ T cells were cultured in the presence of 500 nM PTEN inhibitor (VO-OHpic) for 6 days with inhibitor added at the indicated days. Shown is the percentage of cells that developed into iTregs (CD25^hi, CD45RA^-, FoxP3^hi, CTLA4^+ and CD127^). (B) Purified human CD4+ T cells were cultured in the presence of varying concentrations of PTEN inhibitor VO-OHpic. Inhibitor was added at the indicated concentration on day 0 and left in culture for 6 days. The percentage of cells that developed into iTregs were quantified as in A.
Figure 5.8: No difference in SHIP1 expression in iTregs

(A) Regulation of gene expression. High levels of SHIP1 gene expression in iTregs compared to other T cell populations can be visualized. GAPDH (housekeeping gene) was assessed as control. (B) Lysates from $3 \times 10^5$ sorted naïve, memory, effector or iTreg cells were analyzed by Western blot to detect the pattern of selected protein expression. Protein band densities were quantified by densitometry and normalized to naïve. (C) Protein band densities were quantified by densitometry using Western blots in B. Ratio of total SHIP1:GAPDH are shown. Data are representative of 3 independent donors.
5.2.4 Kinase activity difference in iTreg TCR signaling

AKT is phosphorylated by mTOR at S473 and PDK1 at T308 (74, 77, 78). We found a trend of increased mTOR protein expression in iTregs compared to naive and memory cells, however it was not significant across all human donors (Figure 5.9A, B, C). mTOR gene expression also remained at the same level in all T cell types (Figure 5.5A). mTOR protein complex formation is required for mTOR activity and two different mTOR complexes can be formed: mTOR complex 1 (mTORC1) or mTOR complex 2 (mTORC2). mTORC2 is involved in phosphorylating AKT at S473, whereas mTORC1 is directly phosphorylated by AKT and is part of a negative feedback loop that inactivates the AKT pathway upstream of AKT. The activity of the mTOR complexes is linked to the phosphorylation of mTOR at the auto-phosphorylation residue S2481 (231, 232). We utilized P-mTOR S2481 levels as a measure of intrinsic mTOR activity in iTregs and identified significantly decreased levels of P-mTOR S2481 upon CD3 stimulation in iTregs compared to mock stimulation, while there was no change in P-mTOR S2481 levels detected in naïve and memory cells (Figure 5.9D, E). These results suggested that upon TCR stimulation, a phosphatase decreases mTOR’s activity. Indeed, pervanadate treatment inhibited the decrease in P-mTOR S2481 levels, implying that this difference was caused by mTOR de-phosphorylation by an unknown phosphatase following TCR stimulation (Figure 5.9D). Quantification of TCR-induced P-mTOR S2481 MFI over pervanadate-treated P-mTOR S2481 MFI is shown in Figure 5.9E. A significant decrease in P-mTOR S2481 was detected in iTregs compared to naïve and memory T cells. To ensure that this effect was specific to the enzymatically active residue and not simply a
general decrease in the phosphorylation of mTOR in iTregs, we examined the levels of mTOR phosphorylation by determining the extent of P-mTOR S2448. There was no significant difference in the P-mTOR S2448 upon TCR stimulation (Figure 5.9F) indicating that the differences we see in P-mTOR S2481 could be specific to that residue which conveys mTOR activity.

Others have reported that the phosphatase PP2A could regulate mTOR activity (233, 234). We decided to determine if PP2A is responsible for mTOR inactivation in response to TCR stimulation in iTregs. To do this, we cultured CD4+ T cells in iTreg polarizing media, rested them for 5-6 hours on ice and then activated them by CD3 crosslinking in the presence or absence of a PP2A inhibitor (okadaic acid). To test the efficacy of okadaic acid, we activated conventional cells in the presence of PP2A inhibitor and found that the levels of P-mTOR S2481 decreased, suggesting that the inhibitor is having an effect on mTOR activity (Figure 5.6). We found that the levels of P-mTOR S2481 did not increase in response to CD3 activation but rather decreased slightly while PP2A was inhibited, suggesting that PP2A is not the phosphatase regulating mTOR in iTregs (Figure 5.10A). Additionally, we added the PP2A inhibitor to our cell culture system to see if inhibiting PP2A affected the development of iTregs. We found that there is no difference in the ability to generate iTregs if PP2A is inhibited (Figure 5.10B), indicating that PP2A is not involved in iTreg development.

Although we found that mTOR was less active (phosphorylated) in response to iTreg TCR engagement we also wanted to evaluate the contribution of additional kinases to the AKT activation blockade. PI3K is the kinase that phosphorylates PtdIns (4,5)P2
and, with lower efficiency, PtdIns 4P, at the 3’-OH position of the inositol ring to produce PtdIns (3,4,5)P₃ and PtdIns (3,4)P₂, respectively thus leading to translocation of AKT to the membrane. PI3K is composed of a regulatory subunit and a catalytic subunit. The regulatory subunit of PI3K can be composed of p85α, p85β or p55γ and will provide the PI3K with its specificity for its substrate. The catalytic subunit of PI3K can be composed of p110α, p110β or p110δ. We found that in iTregs, the levels of protein and gene expression of the regulatory subunit most often found in PI3K, p85α, were the same compared to other T cell populations (Figure 5.11A, B). Interestingly, while naïve and memory T cells do not express detectable levels of p55γ, iTregs express detectable, significantly higher gene and protein levels (Figure 5.11A, B). Moreover we found that the levels of p110β were the same in all T cell populations examined (Figure 5.11C). These results suggest that PI3K may target alternative substrates in iTregs compared to conventional T cells due to its expression of the p55γ regulatory subunit in addition to p85α.

PDK1 is one of the two kinases that phosphorylate AKT leading to its activation. We identified that PDK1 gene expression is lower in iTregs than other T cell populations (Figure 5.12A). However, PDK1 protein expression was significantly higher in iTregs (Figure 5.12B, D). The levels of PDK1 also correlated with the increased levels of P-PDK1 in iTregs compared to other T cell populations (Figure 5.12B, D). Quantification of PDK1 protein expression is shown in Figure 5.12C. Upon CD3 activation the level of P-PDK1 slightly decreased in all T cell populations (Figure 5.12 D, E), suggesting that
PDK1 activity is not augmented in iTregs compared to conventional T cells and is not likely a signaling molecule involved in the blockage of AKT activity.
Figure 5.9
Figure 5.9

D

Naïve  Memory  iTreg  iTreg

@-mTOR S2481

Mock
CD3 X
Pervanadate

E

@-mTOR S2481 Index

Naive  Memory  iTreg

F

@-mTOR S2481 Index

Naive  Memory  iTreg
Figure 5.9: mTOR is inactivated upon TCR stimulation in iTregs

(A) Purified iTregs selectively express mTOR at levels equal to other T cell populations. Lysates from $3 \times 10^5$ sorted naïve, memory, effector or iTreg cells were analyzed by Western blot to detect the pattern of selected protein expression. Protein band densities were quantified by densitometry. (B) Protein band densities were quantified by densitometry using Western blots in A. Ratios of total mTOR:GAPDH are shown. Data were analyzed for significance using a repeated measures ANOVA followed by a Student Newman-Keuls multiple comparisons post-hoc analysis. Data are representative of 4 independent donors. (C) mTOR Expression was assessed by flow cytometry in naïve, memory, and iTreg subsets. (D) Cultured human T cells were rested on ice for 5-6 hours and then stimulated with CD3 or pervanadate for 3 minutes. Flow cytometry was performed for phospho-AKT S473, CD25, CD45RA, FoxP3 and CTLA4. (E) Quantification of data in D was analyzed using the CD3 activation index of P-mTOR S2481 in T cells measured by flow cytometry. The P-mTOR S2481 index was determined by taking the percentage of MFI of P-mTOR S2481 following CD3 activation and dividing it by MFI P-mTOR S2481 after pervanadate treatment (representing the maximum potential of P-mTOR S2481). Data were analyzed for significance using a repeated measures ANOVA followed by a Student Newman-Keuls multiple comparisons post-hoc analysis. **p<0.01. *p<0.05. These data are representative of 6 independent human donors. (F) The P-mTOR S2448 index was defined as the percentage of MFI of P-mTOR S2448 upon CD3 activation divided by MFI P-mTOR S2448 after pervanadate treatment (representing the maximum potential of P-mTOR S2448). Data were analyzed for significance using a repeated measures ANOVA followed by a Student Newman-Keuls multiple comparisons post-hoc analysis. This data is representative of 5 independent human donors.
Figure 5.10: PP2A is not the phosphatase regulating mTOR

(A) Cultured human T cells were rested on ice for 5-6 hours then treated with a PP2A inhibitor (okadaic acid) for 30 minutes at 37°C. The cells were then stimulated with CD3 for 3 minutes. Flow cytometry was performed for phospho-mTOR S2481, CD25, CD45RA, FoxP3 and CTLA4. iTregs were identified by their phenotype of high expression of CD25, FoxP3, CTLA4 and CD45RA negative. MFI of P-mTOR S2481 is shown following CD3 activation in iTregs. Inhibiting PP2A does not appear to effect the levels of P-mTOR S2481. Data are representative of three independent donors. Data were analyzed by Student’s t-test. Error bars represent SEM. Representative of 3 independent donors.

(B) Purified human CD4+ T cells were cultured for 6 days with vehicle or the inhibitor of PP2A (1nM okadaic acid) added at the indicated day. After 6 days in culture, the cells were analyzed for the percentage of cells that developed into iTregs based on criteria above. Error bars represent SEM. Representative of 3 independent donors. Adding PP2A inhibitor to our culture system did not affect the development of iTregs.
**Figure 5.11: iTregs express significant amount of p55γ**

(A) Regulation of gene expression. High levels of p55γ gene expression were found in iTregs compared to other T cell populations, however p85α remained the same throughout each T cell population. GAPDH (housekeeping gene) and FoxP3 (positive control) were assessed as controls. (B) Purified iTregs selectively express high levels of p55γ protein. Lysates from 3 x 10^5 sorted naïve, memory, effector or iTreg cells were analyzed by Western blot to detect the pattern of selected protein expression. The same membrane was sequentially stripped and reprobed. Protein band densities were quantified by densitometry. (C) Purified iTregs express relatively identical levels of p110β protein to other T cell populations. Lysates from 3 x 10^5 sorted naïve, memory, effector or iTreg cells were analyzed by Western blot to detect the pattern of selected protein expression. Protein band densities were quantified by densitometry.
Figure 5.12

E

Naïve  Memory  iTreg

\&-PDK1

F

\&-PDK1 Index

**

Naïve  Memory  iTreg
Figure 5.12 PDK1 activity is not diminished in iTregs
(A) Regulation of gene expression. Lower levels of PDK1 gene expression were found in iTregs compared to other T cell populations. GAPDH (housekeeping gene) was assessed as control. (B) Purified iTregs selectively express higher levels of PDK1 compared to other T cell populations. Lysates from 3 x 10⁵ sorted naïve, memory, effector or iTreg cells were analyzed by Western blot to detect the pattern of selected protein expression. Protein band densities were quantified by densitometry. (C) Protein band densities were quantified by densitometry using Western blots in B. Ratio of total PDK1 and phospho-PDK1:GAPDH are shown. Data were analyzed for significance using a repeated measures ANOVA followed by a Student Newman-Keuls multiple comparisons post-hoc analysis. N=3-5 independent donors. (D) PDK1 and P-PDK1 levels were assessed by flow cytometry in naïve, memory, and iTreg subsets. (E) Cultured human T cells were rested on ice for 5-6 hours then stimulated with CD3 or pervanadate for 3 minutes. Flow cytometry was performed for phospho-PDK1, CD25, CD45RA, FoxP3 and CTLA4. (F) Quantification of data in E was analyzed using the CD3 activation index of P-PDK1 in T cells measured by flow cytometry. The P-PDK1 index was defined as the percentage of MFI of P-PDK1 upon CD3 activation divided by MFI P-PDK1 after pervanadate treatment (representing the max potential of P-PDK1). Data were analyzed for significance using a repeated measures ANOVA followed by a Student Newman-Keuls multiple comparisons post-hoc analysis. **p<0.01. *p<0.05. These data are representative of 4 independent human donors.
5.2.5 AKT associates with SMAD3 and regulates SMAD3’s transcriptional activity in iTregs

The accumulation of significant levels of inactive AKT suggests a possible physiologic role for its inactive form. There is evidence to suggest that, in some cell types, AKT binds to the transcription factor SMAD3 (235-237). This was particularly intriguing to us because TGF-β signaling, which activates SMAD3, is required during iTreg development (182). Upon phosphorylation, SMAD3 becomes activated and translocates into the nucleus where it regulates transcription, typically upregulating pro-apoptotic factors. However, SMAD3 upregulates the expression of FoxP3 in iTregs, implying that SMAD3 activity is critical during iTreg differentiation (187, 238). iTregs express significantly more SMAD3 protein than other T cell populations, which coincided with an increase in the P-SMAD3 levels (Figure 5.13A, B), indicating a robustly activated TGF-β signaling pathway in iTregs. These data provide a possible mechanism for upregulated FoxP3 expression by SMAD3 during iTreg development (187, 238). To determine if AKT-SMAD3 association occurs in human iTregs, we immunoprecipitated AKT and then probed for SMAD3. We observed more AKT and SMAD3 association in iTregs than in conventional T cells (Figure 5.14A). Confirmation of this relationship was obtained by reversing the immunoprecipitation (Figure 5.14B). We have determined that AKT localizes to the membrane and have demonstrated a unique interaction between AKT and SMAD3. Given that SMAD3 must be activated at the membrane and translocate to the nucleus to regulate transcription, we next sought to investigate the location of AKT-SMAD3 interaction. iTregs and conventional T cells were stained with AKT and SMAD3 antibodies in order to examine their intracellular
location via confocal microscopy. AKT and SMAD3 appeared to colocalize in the cytoplasm of iTregs (Figure 5.15), suggesting that AKT-SMAD3 association could be negatively regulating SMAD3 transcriptional activity by inhibiting its translocation into the nucleus. Conventional T cells and iTregs were transfected with a SMAD3 reporter plasmid (pGL3 Basic CAGA12 Luc) (192) containing a series of CAGA repeats linked to a luciferase reporter which allowed indirect measurement of SMAD3 activity through luminescence (Figure 5.16A). We found that conventional T cells had significantly higher SMAD3 activity compared to iTregs (Figure 5.16B), indicating that AKT-SMAD3 association regulates SMAD3 transcriptional activity. These results illustrate a new, non-enzymatic function of AKT in iTregs by way of an AKT-SMAD3 interaction that inhibits SMAD3 activity, and has revised our view of the signaling events occurring in iTreg development. We hypothesize that iTreg development involves a two-step process (Figure 5.17), in which T cells first undergo a significant round of proliferation that requires high levels of AKT activity and rapid AKT turnover with concomitant FoxP3 induction by SMAD3 (Figure 5.17). Once divided, the cells then undergo rewiring of their signaling network which leads to differentiation of the T cells into iTregs. Subsequent blockage of AKT activity results in AKT accumulation in the cytoplasm which negatively regulates SMAD3 activity by preventing SMAD3 translocation into the nucleus via its physical association with AKT.
Figure 5.13: iTregs express high levels of SMAD3

(A) Total protein expression was analyzed using flow cytometry for SMAD3 in various T cell subsets. Quantification of SMAD3 MFI from 4 independent human donors. (B) Phospho-SMAD3 expression was assessed by flow cytometry in naïve, memory, and iTreg subsets. Quantification of P-SMAD3 MFI from 4 independent human donors. Data were analyzed for significance using a repeated measures ANOVA followed by a Student Newman-Keuls multiple comparisons post-hoc analysis. *p<0.05.
Figure 5.14

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SMAD3

AKT

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Ratio of SMAD3 Bound/AKT

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SMAD3

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Ratio of AKT Bound/SMAD3
Figure 5.14: SMAD3 associates with AKT in iTregs

(A) iTregs which were induced in optimization iTreg polarizing media and conventional T cells were immunoprecipitated with agarose beads bound to AKT antibody. The immunoprecipitate was then run on an SDS-PAGE gel and analyzed via Western blot. Blots were probed for SMAD3 or AKT. The ratio of SMAD3/AKT was calculated using densitometry measurements of protein expression. (B) iTregs and conventional T cells were immunoprecipitated with agarose beads bound to SMAD3 antibody. The immunoprecipitate was then run on an SDS-PAGE gel and analyzed via Western blot. Blots were then probed for AKT or SMAD3. The ratio of SMAD3/AKT Bound was calculated using densitometry measurements of protein expression.
Figure 5.15: SMAD3-AKT association occurs in the cytoplasm of iTregs
Confocal microscopy was performed on sorted naïve, memory, effector and iTregs. Cells were stained for total AKT (green) and SMAD3 (red) protein, plus DAPI (blue).
Figure 5.16

A

CAGA Box

AGCCAGACA

Hind III

MLP

1 2 3

12

Xho I

SMAD3 x 12

Luciferase

pGL3-Basic Vector backbone

(CAGA)_{12} MLP-Luc

Amp^R

B

Relative Luciferase (Arbitrary Units)

Conv. iTreg

2.5 *

2.0

1.5

1.0

0.5

0.0

151
Figure 5.16: SMAD3 transcriptional activity

(A) Investigation of SMAD3 transcriptional activity was performed using the pGL3 Basic MLP (CAGA)\textsubscript{12} Luciferase plasmid, which contains a SMAD3 CAGA box repeated 12 times linked to a luciferase reporter. SMAD3’s transcriptional activity induces luciferase production in the cells.

(B) PHA-stimulated (effector cells) and iTreg cells which were cultured in our optimization iTreg polarizing media were transfected with a pGL3 Basic MLP (CAGA)\textsubscript{12} Luciferase plasmid (SMAD3 Activity Reporter) and pGLRP (Transfection control) at day 5, lysed and analyzed for Luciferase and Renilla levels. Relative Luciferase units are luciferase/renilla. Two tailed paired student’s t test was performed * p<0.05.
iTreg development may consist of two main steps. CD4⁺ T cells first undergo a significant round of proliferation in culture, during which AKT is highly active and there is a rapid AKT protein turnover in the cells. At this early stage, SMAD3 signaling induces FoxP3 induction in the T cells. Once divided, the cells then undergo a rewiring of signaling networks which leads to differentiation of the T cells into iTregs. There is a blockage of AKT activity in iTregs due to these changes in signaling leading to an accumulation of AKT protein in the cytoplasm of the cells. AKT-SMAD3 then associate with each other in the cytoplasm of the cells, thereby regulating SMAD3’s transcriptional activity.
5.3 Summary

We have demonstrated a blockage of the TCR-dependent AKT activation in human iTregs also present in other regulatory T cell subsets that could be critical for regulatory T cell development and function. Moreover, we have shown that this blockage of AKT activity is not due to a general failure of AKT activation, but appears to be TCR specific. AKT accumulates in iTregs most likely due to its inability to become activated and subsequently degraded. We set out to determine the cause of AKT activity blockage in iTregs. We found that AKT was recruited to the membrane upon TCR stimulation and localizes to lipids rafts. iTregs have a different protein composition in lipid rafts compared to conventional T cells suggesting that the defective AKT signaling could be due, at least in part, to the inability to form functional protein complexes. In addition to intracellular localization, several kinases and phosphatases are crucial to the activation and inactivation of AKT in the TCR signaling network. iTregs exhibit an overexpression of the phosphatase PTEN compared to conventional T cells, however inhibition of PTEN resulted in no effect on the differentiation of iTregs. Our data suggest that PTEN is involved in TCR signaling when CD3 and CD28 are stimulated, but not when CD3 is stimulated alone. Although the overexpression of PTEN could be critical for inhibiting TCR AKT activity following CD3-CD28 costimulation, we do not use CD28 for development of human iTregs. Thus, inhibiting PTEN has no effect on the ability to generate iTregs in our culture system. Additionally, we found that there is no difference in the expression of SHIP1 in iTregs compared to conventional T cells, precluding the role of SHIP1 in differential iTreg AKT pathway signaling.
Having found no evidence to support the involvement of differential intracellular AKT localization, PTEN, or SHIP1 in the inactivation of AKT in iTregs, our attention then turned to the kinases that activate AKT. As mTOR is known to induce AKT activation through phosphorylation, our observation of decreased mTOR activity upon TCR stimulation in iTregs suggests that this could be a key step in the blockage of AKT activity. Treatment with a phosphatase inhibitor (pervanadate) blocked the TCR induced inhibition of mTOR activity, suggesting mTOR is inactivated by a phosphatase. PP2A has been implicated as the phosphatase regulating mTOR’s activity by others (233, 234), however pharmacologic inhibition of PP2A did not increase TCR induced mTOR activity in our hands. Which phosphatase regulates mTOR activity in iTregs remains unclear. Additionally, PDK1 and P-PDK1 levels were increased in iTregs, suggesting that AKT blockage does not result from decreased PDK1 activity. Lastly, we found that PI3K subunits in iTregs are differentially expressed. Human iTregs exhibit significantly more of the regulatory subunit p55γ than conventional T cells, indicating that PI3K may have different as yet unidentified substrates in iTregs, thus making it an attractive target for further study. These results suggest that the TCR-induced blockage of AKT activity is not necessarily caused by one signaling molecule, but is the result of a combination of differential regulatory events in the TCR pathway leading to blockage of AKT.

Having identified several key signaling changes that could lead to TCR-induced AKT inhibition, we next wanted to investigate the role of AKT itself in iTregs. We demonstrated that AKT accumulates in iTregs which leads to AKT-SMAD3 association. AKT-SMAD3 association occurs in the cytoplasm, indicating it is regulating SMAD3’s
transcriptional activity. SMAD3 activity was significantly lower in iTregs despite the overexpression of SMAD3 protein and higher P-SMAD3 levels. Though we did not examine TGF-β induced SMAD3 independent signaling, its role in iTreg development cannot be discounted by our studies and further investigation of SMAD3 independent signaling is warranted. We hypothesize that the AKT-SMAD3 association is a critical late step in iTreg development, due to the fact that SMAD3 signaling is required during the early iTreg development to induce FoxP3 expression, yet overactivation initiates apoptosis. These studies demonstrate the attractiveness of the AKT-SMAD3 association as a potential target for modulating iTreg development in the treatment of disease.
Chapter 6: Discussion

6.1 Overcoming the deficiency in generating and studying iTregs

The critical importance of Tregs in the human immune system is made evident by the numerous diseases associated with their dysregulation (17). iTregs comprise the majority of the circulating peripheral Tregs in the human body (25). It has been shown that transfer of Tregs into mice is beneficial in murine models of autoimmune diseases and/or chronic inflammatory diseases like colitis (194), type I diabetes (42), RA and graft versus host disease (195). However, current limitations, such as inadequate number, the fidelity of Treg homing specificity, and insufficient purity and stability, must be overcome before the manipulation of Tregs can serve a useful therapeutic role. Prior to the work outlined in this dissertation, we were unaware of any cell culture systems available that could generate large amounts of human iTregs \textit{ex vivo}. Therefore, our lab has established an innovative \textit{ex vivo} cell culture system that mimics \textit{in vivo} iTreg development (188). This system allows for the discrimination and comparison of naïve, memory and iTreg T cell populations simultaneously within a single donor over the course of six days in culture. Cells that differentiate into iTregs arise from a highly proliferative population of T cells that start to mature around day three in culture and then progressively increase in quantity and expression of iTreg-specific cell markers over the next several days until 20-35\% of cells have differentiated into iTregs. Optimization of iTreg development results in differentiation of approximately 80\% of the cells into iTregs. Human iTregs exhibit high levels of FoxP3, CTLA4, GITR and are CD127
negative. Comprehensive examination of human iTreg phenotype revealed expression of CD86, a marker usually expressed by APCs, and not expressed on other T cell subsets, may facilitate an iTreg’s interaction with other T cells. Additionally, our finding that CCR2 and CCR1 are present on human iTregs suggests they can traffic to sites of inflammatory chemokine production. iTregs also exhibit high levels of CD58, which when bound to its ligand CD2 optimizes immune recognition and T cell expansion (202). CD58/CD2 ligation strengthens the interaction between a Treg cell and a target conventional T cell (203), which may be critical for efficient suppression. CD30, a marker of activated T cells, was displayed on iTregs as well, and has been proposed to be involved in the regulatory function of Tregs in vivo (205). Human iTregs have reduced levels of CD31 normally expressed on naïve cells implying they may undergo a change in the pattern of migration in vivo. CD31 is a major constituent of the endothelial cell intracellular junction. The expression of CD31 on the cell surface of T cells has been shown to modulate their transmigration across endothelial cell barriers (239-241). These results suggest that human iTregs may possibly be trafficking to different areas in vivo compared to naïve T cells likely by exploiting a mechanism other than CD31. Lastly, human iTregs have a higher expression of Galectin 3 compared to effector T cells (Fig. 5.4). Galectin 3 is known to prevent small microclusters on the membrane to form active macrocomplex (228). Therefore, iTregs may have an inability to form large macrocomplexes necessary for optimal TCR-mediated signal initiation upon TCR stimulation. The expression pattern of these phenotypic markers is consistent with Tregs. In the presence of iTregs, effector T cell proliferation dramatically decreased confirming
the suppressor ability of the iTregs generated by our culture system. In addition, our data suggest that iTreg suppression appears to be mediated through both cell-to-cell contact and secretion of soluble factors, such as IL-10.

Together, these data create a working model of iTreg development. We hypothesize that iTregs develop from CD4+ T cells via a two-step process. First, CD4+ T cells must undergo a substantial round of proliferation, followed by the rewiring of their TCR signaling network which subsequently induces transformation of CD4+ T cells into iTregs.

6.2 TCR proximal signaling events involved in iTreg development

A better understanding of the basic biology of iTregs is the first step in their development as a therapeutic treatment. One of the earliest known events in iTreg signaling is the failure to activate the PI3K/AKT pathway (145). Constitutive activation of AKT inhibits the differentiation of T cells into iTregs through the blockage of FoxP3 induction and, additionally, leads to the loss of suppressor function in nTregs (146, 147). These results support the need for AKT that is “silent” in order to sustain Treg development and function. However, the specific mechanism by which AKT activation is blocked remains unknown.

We examined the events upstream and downstream of AKT following TCR stimulation to elucidate the events surrounding blockage of AKT. We demonstrated partial activation of the TCR signaling pathway in iTregs compared to conventional T cells and the failure of AKT to become activated upon TCR induction in iTregs. In contrast, upon crosslinking with anti-CD3, ERK activation is highly effective in iTregs,
suggesting that the blockage of AKT activity and the selective activation of TCR are not secondary to a general failure of TCR signaling; rather, these are events specific to the AKT pathway.

iTregs also exhibited high levels of active Lck at rest that did not increase upon TCR ligation, independent of total Lck protein or gene expression. These data indicate that Lck may be activated by an alternative signaling pathway in iTregs. iTregs exhibit high expression of CD25, the IL-2Rα subunit which is expressed on activated and regulatory T cells, which generates a high affinity IL-2R complex. We observed the association of Lck with the highly active IL-2R upon IL-2 treatment in iTregs. Lck is a key proximal molecule in TCR signaling that initiates the downstream events that could direct AKT inactivation in iTregs. The first demonstration of the uncoupling of Lck from the TCR involved its relocation from TCRβ to CD3 ε-chain to regulate calcium flux in thymocytes and lymph node T cells, and was later found to relocate to CD45, CD4 and CD8 in mature primary T cells (242-245). The displacement of Lck to the high affinity IL-2R has also been demonstrated in a mouse CD4+ T cell clone where Lck functionally uncouples from the TCR signaling machinery through diversion of the subcellular localization of Lck to the IL-2R multiprotein (222). This finding suggests that there is a pool of Lck that can localize from the TCR to the IL-2R and may provide another level of iTreg TCR regulation.

Further downstream of Lck, we found only partial LAT phosphorylation in iTregs, which is concordant with our observation of partially decreased PLCγ phosphorylation, suggesting TCR-induced Ca2+ flux may also be negatively affected.
Kv1.3 is critical for hyperpolarizing the plasma membrane in response to the change in potential following TCR-induced Ca^{2+} flux (161). Kv1.3 levels were the same throughout all T cell populations we examined, however Kv1.3 activity was very weak in iTregs following TCR ligation, indicating a functional dissociation between TCR and Kv1.3. Lck has been shown to be functionally linked to the Kv1.3 channel during the formation of the immune synapse (216-218). Therefore, because Lck relocates to the IL-2R, Kv1.3 could relocate with it. Our finding that Kv1.3 is activated in response to IL-2 in iTregs provides evidence of a possible mechanism for the inhibition of AKT through a functional uncoupling of key proximal signaling molecules from the TCR and their relocation to the highly active IL-2R in iTregs.

As we determined that Kv1.3 relocates to the IL-2R in iTregs, we next examined the integrity of Ca^{2+} flux in response to TCR stimulation in iTregs. Ca^{2+} flux remained intact in iTregs, suggesting compensation for the lack of Kv1.3 activity at the TCR by another ion channel. KCa3.1 was subsequently found to be overexpressed in iTregs, and upon its inhibition Ca^{2+} flux was suppressed, suggesting KCa3.1 may be responsible for regulating the TCR-induced Ca^{2+} flux in iTregs. These data implicate a regulatory switch of K^{+} channel activity in iTregs that may constitute a key contributing factor in signaling rewiring associated with the development of human iTregs. These novel findings indicate an even higher level of TCR receptor regulation in iTregs, where the movement of protein complexes from the TCR is critical for differentiation. This complex reorganization is more than likely a key event, the understanding of which will be important in future iTreg signaling studies. We hypothesize that the interplay between the
IL-2R and the TCR may also be shared by other cytokine pathways. IL-15 is a cytokine that is structurally similar to IL-2 and signals through a complex composed of the IL-2Rβ chain and the common γc chain (246). IL-15 is critical to providing survival signals that maintain memory T cells in the absence of antigen. We found that Lck associates with the IL-2Rβ chain in iTregs, therefore it is possible that Lck and Kv1.3 can also relocate to the IL-15R in memory T cells, and this relocation may be critical for maintaining survival of the memory T cells. These studies shed light on the value of understanding the regulation of functional uncoupling of protein complexes from the TCR and the cross talk between cytokine pathways.

Aside from AKT pathway inactivity, very little was known about iTreg-specific signaling events prior to the studies outlined in this dissertation. We found that key signaling events upstream of AKT are relocated to the highly active IL-2R in iTregs and have provided new insight into the targeting of Kv1.3 channels for the treatment of autoimmune disease. Studies are underway targeting T_{EM} cells in MS patients through inhibition of the Kv1.3 channel (166). These studies have found that inhibiting Kv1.3 channels helps to control the pathogenicity of T_{EM} cells, however given our findings that Kv1.3 is located at the IL-2R in iTregs and that it may be critical in maintaining a steady population of peripheral Tregs, targeting Kv1.3 could lead to the loss of iTreg function, thus exacerbating the disease. Special consideration should be given to the effect of Kv1.3 inhibition on iTreg function when modulating its function in a therapeutic setting in vivo.
Other potential applications for inhibiting Kv1.3 would be in a mixed T cell culture *ex vivo*, in which Kv1.3 inhibition may help select a pure population of iTregs ready for re-infusion into an autologous donor. One of the hurdles that must be overcome before iTregs can be used for therapeutic treatment is the ability to generate a pure population of iTregs. This task is made difficult by the phenotypic similarities between iTregs and effector T cells, which can also be generated during *ex vivo* expansion. As it is known that Kv1.3 activation is a necessary step in effector T cell activation but is no longer functionally linked to the TCR in iTregs, one possible solution to this problem would be to inhibit Kv1.3 to preferentially or selectively prevent TCR-dependent effector T cell proliferation and facilitate the generation of a pure population of iTregs *ex vivo*.

### 6.3 TCR distal signaling events involved in iTreg development

Our investigation turned to the differential regulation of kinases and phosphatases that control AKT’s activity further downstream of the TCR. AKT protein expression was shown to be significantly greater in iTregs compared to conventional T cells, however AKT transcript levels were the same compared to other T cell populations. Additionally, though there were higher levels of AKT in iTregs, its activity did not increase in response to TCR stimulation, suggesting that AKT accumulation in iTregs is most likely because of decreased degradation. We found that AKT translocates to the membrane upon TCR stimulation in iTregs, implying that AKT blockage occurs after its recruitment to the membrane.

PTEN and SHIP1, phosphatases that regulate the level of PtdIns (3,4,5)P$_3$ upon TCR induction in T cells, were evaluated in iTregs. We uncovered no difference in the
expression of SHIP1 in iTregs compared to conventional T cells, suggesting that SHIP1 may not have a role in differential AKT pathway signaling in iTregs. However, this should be examined in a functional assay to completely rule out this possibility. Although human iTregs express higher levels of PTEN compared to effector T cells, its inhibition did not affect the development of iTregs in our system. Additionally, our data suggest that PTEN is involved in TCR signaling when CD3 and CD28 are stimulated, but not under CD3 stimulation alone. Though PTEN overexpression could be critical for inhibiting TCR-induced AKT activity following CD3-CD28 costimulation in other systems or in vivo, its inhibition had no effect in our system, as we do not need CD28 for iTReg induction. The discrepancy could be explained by the signaling pathways initiated by CD3 and CD28 crosslinking, along with the function of the cell types that need both signaling molecules. Naïve T cells require TCR/CD3 stimulation along with costimulation through CD28 to develop, activate and differentiate into a conventional T helper cell (247). CD28 co-stimulation induces the production of IL-2, increasing T cell metabolism, proliferation and differentiation through the strong activation of the AKT pathway (247). Memory T cells, however, only need CD3 stimulation to become active and lead to AKT activation, but to a lesser extent. In culture conditions where α-CD28 is not included, we believe the iTregs generated develop primarily from memory T cells, and that PTEN may not play a role in TCR-dependent AKT pathway regulation. However, when iTregs are generated from naïve cells in media containing α-CD28, a strong AKT activation unfavorable to iTreg development could be induced. In this
instance, PTEN regulation is crucial to dampen the response inhibiting AKT activation at the CD28 receptor.

Another potential regulatory role for PTEN overexpression in iTregs could be linked to the observation by others that PTEN inhibits IL-2R mediated expansion of Tregs, and that this regulation by PTEN is important for peripheral homeostasis in murine Tregs in vivo (174). This is interesting to us as we have already established that other key proximal signaling molecules, Lck and Kv1.3, translocate to the IL-2R in human iTregs. Thus, the possibility exists that PTEN is also active at the IL-2R in human iTregs; however, further investigation is needed for confirmation of this hypothesis.

Activation of AKT occurs through phosphorylation by mTOR at S473 and PDK1 at T308 (74, 77, 78). We identified a trend of increased mTOR protein expression in iTregs compared to naïve and memory cells, in some, but not all human donors. Interestingly, upon CD3 stimulation, phospho-mTOR levels decreased in iTregs, suggesting that mTOR inhibition in response to TCR activation could lead to blockage of AKT activity. These data fit very well with previous data using the mTOR inhibitor rapamycin, an immunosuppressive drug currently approved to prevent organ rejection, especially useful in kidney transplants. Rapamycin enables the expansion and induction of murine and human Tregs (248-250) and highly increases human iTreg suppressor activity in both in vitro and in vivo (248). Combined, these data highlight the necessity of mTOR inhibition in Tregs and suggest another phosphatase in the regulation of mTOR’s activity in iTregs after TCR stimulation. PP2A is a phosphatase known to regulate mTOR activity (233, 234). However, inhibition of PP2A activity with okadaic acid did not result
in increased mTOR activity in iTregs. Together, these findings identify mTOR inhibition as a crucial step in the differentiation of iTregs, and accentuate the need to identify the phosphatase responsible for its regulation.

AKT is also activated in response to TCR stimulation by another kinase, PDK1. PDK1 protein expression is increased in iTregs compared to conventional T cells, which correlates with an increase in its phosphorylated form. These data suggest that PDK1 activity does not diminish in iTregs, and is unlikely to lead to the blockage of AKT activity. However, increased PDK1 protein expression could compensate for the lack of AKT activity in iTregs, as the downstream targets of PDK1 and AKT appear to overlap (251). Confirmation of this compensatory relationship will require further investigation.

AKT recruitment to the membrane requires phosphorylation of phosphoinositols by the kinase PI3K. When PI3K expression was examined in iTregs, we found substantially higher incorporation of the p55γ subunit responsible for recruiting PI3K to its targets, versus conventional T cells. These data suggest that PI3K may have different downstream targets in iTregs than in conventional T cells, leading to a different signaling profile downstream of PI3K and subsequent rewiring of signaling pathways. It is important to note that we did not find a difference in the ability of PI3K to recruit AKT to the membrane upon TCR stimulation, indicating that PI3K still activates phosphoinositols and that it produces enough PtdIns (3,4,5)P₃ to recruit AKT to the membrane, but could also induce other signaling pathways. A definitive role for PI3K in AKT recruitment to the membrane will be necessary for further understanding of this signaling pathway.
Having identified several key signaling molecules that lead to the inactivation of AKT in iTregs, we next set out to examine the role of AKT itself in iTreg differentiation. Significantly higher AKT levels suggest a physiologic function for AKT within the cell during iTreg development. Others have shown that AKT associates with the transcription factor SMAD3 in various cell lines (235-237) and that, in some instances, the AKT-SMAD3 association regulates AKT activity while in others, SMAD3 activity is affected (235-237). This was intriguing to us as TGF-β signaling, which is responsible for SMAD3 activation, is necessary for iTreg development. We found significantly more SMAD3 and phospho-SMAD3 in iTregs compared to conventional T cells suggesting an abundance of both AKT and SMAD3 may be leading to their interaction in iTregs. Interestingly, AKT was identified to associate with SMAD3 in the cytoplasm of iTregs. SMAD3 activity was significantly decreased in iTregs compared to conventional T cells, implying that AKT-SMAD3 association prevents SMAD3 from translocating into the nucleus of the cell where it can regulate transcription. To the best of our knowledge, these data present the first report of a non-enzymatic function for AKT in human iTregs. We speculate that the AKT-SMAD3 interaction provides temporal regulation critical for iTreg development that allows SMAD3’s induction of FoxP3 early during development, but later inhibits SMAD3’s activity, preventing SMAD3 induced apoptosis. Additionally, we suggest that the AKT-SMAD3 association is a novel target for the modulation of iTreg development and maintenance. The prevention of this protein association can potentially promote a pro-inflammatory response, and could be useful in cancer and some infections which reduce immunosurveillance as part of its evasion strategy.
6.4 Modulation of the AKT pathway in iTreg development in the clinic: from bench discovery to valuable clinical tool

Unlike mouse work or cell culture work with human-derived cell lines that need to be confirmed in humans before use as a human therapeutic, our work is directly translatable as we are working directly with primary human T cells. We have found that the TCR induced AKT pathway is inactivated in iTregs when compared to effector T cells and have elucidated the cause of this blockage. The AKT signaling pathway is integral to cell survival and has been closely associated with development and progression of tumors (252). Therefore, the AKT pathway is widely targeted in cancer therapeutics. However, whether or not the AKT pathway is a beneficial target in the development of iTregs remains to be determined. We hypothesize that targeting the AKT pathway to inhibit iTreg development presents a novel treatment pathway in diseases associated with over-active Tregs. In certain types of cancer, it is thought that Tregs are either recruited to the microenvironment of the tumor or are induced in the microenvironment of the tumor itself (253, 254) and, once there, are facilitating the tumor’s evasion of immune system recognition. If this concept of Treg assistance in tumor concealment is accurate, modulation of iTreg development and maintenance at the site of the tumor would be highly beneficial to cancer patients. We hypothesize that direct modulation of iTreg development through targeting the AKT pathway by specifically inhibiting the colocalization of AKT with SMAD3 will induce apoptosis in iTregs and possibly alleviate the blockage of the immune system tumor recognition.

Targeting the AKT pathway could also be beneficial in cases where there are inherent defects in iTreg generation or function. Mouse models of chronic inflammatory
disease (i.e., IBD, asthma, type I diabetes) have demonstrated that treatment with \textit{ex vivo} Tregs helped ameliorate disease pathology \cite{41}. In humans, large-scale \textit{ex vivo} generation of Tregs through inhibition of the AKT pathway followed by repatriation within the patient could help to ameliorate the disease. Though we have overcome the deficiencies in the ability to generate a sufficient number of iTregs, there are still roadblocks which we must overcome to ensure the viability of a treatment like this. It is essential to confirm that iTregs generated \textit{ex vivo} will not revert back into an inflammatory phenotype, thus worsening the disease. Additionally, we have yet to decipher how iTregs traffic to specific tissues or organs in the body. Patients with JIA or MS have normal functional Tregs, however their effector T cells are resistant to Treg suppression \cite{36, 44}. In this case, modulating the AKT pathway in iTregs would not affect patient outcome as their Tregs appear to be suppressive \textit{ex vivo}. Surprisingly, however, it has been shown that effector T cells found in the joints of JIA patients have an overactive AKT pathway \cite{44}. Therefore, targeting the AKT pathway in effector T cells could possibly reduce the pathologic effects of effector T cell function in JIA patients by decreasing the inhibition of effector T cell suppression by the Tregs in the microenvironment. However, in MS patients the presence of an overactive AKT has not been identified as yet. Overactive AKT in MS patients would need to be verified before a direct hypothesis on how to treat these patients could be formulated. Regardless of the T cell type responsible for pathology, we postulate that targeting the AKT pathway for the modulation of iTreg development is a novel target for therapeutic treatment.
6.5 Future Directions

We have presented evidence herein for a mechanism of the inactivation of AKT in iTregs in which several signaling pathways are reorganized. A relocation of K+ ion channels from the TCR to the IL-2R in iTregs and a regulatory switch to KCa3.1 usage could regulate TCR-induced Ca^{2+} flux in iTregs, however confirming these results through high-throughput patch clamp technology will be necessary to definitively establish KCa3.1’s role. It will be important to understand the functional role of Kv1.3 at the IL-2R and whether or not it is fundamental to the maintenance and function of Tregs. If Kv1.3 activation is indeed critical for iTreg function, special consideration of its role in iTreg function should be taken into account when using Kv1.3 inhibitors to treat autoimmune disease.

Additionally, we have shown increased expression of the PI3K subunit p55γ in iTregs compared to conventional T cells. We hypothesize that p55γ is critical for iTreg development. PI3K is a critical player in the TCR pathway that will generate PtdIns (3,4,5)P3 subsequently leading to activation of second messengers like IP3 and AKT. In iTregs there is a significantly higher level of expression of p55γ versus naïve cells suggesting that this key signaling molecule may have different downstream targets in iTregs versus conventional T cells which utilizes p85α leading to a differential signaling pattern. A stable knockdown of p55γ in human CD4+ T cells would allow us to determine if p55γ is necessary for iTreg development. After demonstrating the need for p55γ, elucidating the targets of PI3K in iTregs will shed light on the function of p55γ expression and may help to uncover novel signaling pathways important for iTreg differentiation. To investigate the targets of p55γ in iTregs it should be possible to
immunoprecipitate p55γ and p85α in iTregs then run the immoprecipitates on a 2D gel. Differences in p55γ association proteins would suggest a different downstream target which could then be analyzed using mass spectrometry to identify the molecules. Identification of the downstream substrates of p55γ would be an exceptional molecular tool for understanding the differential signaling pathways in iTregs compared to conventional T cells.

mTOR was found to have reduced activity upon TCR stimulation in iTregs, and its inactivation appears to be dependent upon an unknown phosphatase. Identifying this phosphatase would be beneficial in understanding the regulation of mTOR’s activity in iTregs and define a potentially druggable target. Once the identity of this phosphatase is established, its inhibition would allow us to confirm its importance in iTreg generation. There is evidence to suggest that the phosphatase that could be regulating mTOR’s activity in iTregs is protein tyrosine phosphatase (SHP2). Ketroussi et al. described dephosphorylation of mTOR by SHP2 when human T cells were stimulated with a specific antigen (255). Therefore, it is plausible that this could also be occurring in our human iTregs positioning SHP2 as an attractive target for further investigation. PDK1 was also found to be overexpressed in iTregs which could potentially compensate for the loss of AKT activity in response to TCR engagement in some downstream events. AKT has been shown to be important in promoting survival in T cells. However AKT is not activated in response to the TCR in iTregs. Even though there is a blockage of AKT activity in iTregs, the cells are a stable population in culture, suggesting that either there
is a low level of AKT activity or that PDK1 may offset AKT’s loss to induce survival of the cell.

Lastly, we have identified that AKT-SMAD3 associate in iTregs, and this association regulates SMAD3 activity. It will be critical to understand the physical association of AKT and SMAD3 and if this interaction is required for iTreg development and function. To test whether the AKT-SMAD3 interaction is fundamental for iTreg differentiation, we could generate a small molecule that is able to inhibit the ability of AKT to bind to SMAD3 through direct binding site competition. Ideally, this small molecule would have a higher affinity for one or both members of the AKT-SMAD3 complex than the strength of their natural interaction. As we have only seen this interaction occurring in iTregs, the presence of this small molecule should not have a negative effect on the function of other cells in the immune system. SMAD3-AKT interaction has been shown to occur in cell lines in vitro, however this is more than likely due to an artificial system were there was an overexpression of AKT and SMAD3 which may not occur physiologically in the cell. A pilot mouse study to determine if AKT-SMAD3 interaction occurs in other tissue will help illuminate any potential interactions. We could then deliver this small molecule to iTregs in vivo in cases where iTreg localization is impinging on the ability of the immune system to recognize a tumor. Once inside the cell this small molecule should be able to cause AKT and SMAD3 to disassociate relieving SMAD3 transcriptional inhibition and subsequently inducing apoptosis of the cells and allowing the immune system to recognize and respond to the tumor.
6.6 Concluding Remarks

The mechanism of human iTreg development occurs in two steps (Figure 5.17). The first step requires the T cells to undergo a significant round of proliferation in which AKT is highly active and rapidly degraded. Additionally, TGF-β activates SMAD3 to induce the expression of FoxP3 during this sequence. After proliferation, the cells start to differentiate into iTregs, where a rewiring of the signaling pathways occurs subsequently leading to the blockage of AKT and its cytoplasmic accumulation. AKT accumulation will facilitate its association with SMAD3, thus negatively regulating SMAD3’s transcriptional activity. As our results were demonstrated in human T cells, its clinical relevance and translational nature should not be understated. The work in this dissertation has elucidated several novel signaling molecules that are differentially regulated in iTregs compared to conventional T cells and has provided substantial basis for targeting signaling pathways to regulate iTreg development, function and maintenance.
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SCIENTIFIC EXPERIENCE
Research Assistant/Graduate Student
University of Kentucky College of Medicine, Department of Microbiology, Immunology and Molecular Genetics.
Mentor: Francesc Martí, Ph.D.
Dates: August 2007 to December 2012.

Research Intern
University of Alabama Department of Chemistry.
Mentor: Martin Bakker, Ph.D.
Dates: June 2006.

TEACHING EXPERIENCE
Teaching Assistant
University of Kentucky, College of Medicine, Department of Microbiology, Immunology and Molecular Genetics
Supervisor: Carol Pickett, Ph.D.
Dates: August 2009 to October 2009
Course: Immunity, Infection & Disease

Adjunct Faculty
Bluegrass Community and Technical College, Natural Science Division.
Supervisor: Yasemin Congleton, Ph.D.
Dates: January 2011 to December 2011.
Course: Human Anatomy and Physiology I
TEACHING EXPERIENCE - CONTINUED

Faculty
Bluegrass Community and Technical College, Natural Science Division.
Supervisor: Yasemin Congleton, Ph.D.
Dates: January 2012 to present.
Course: Human Anatomy and Physiology I

PUBLICATIONS


ABSTRACTS – asterisk denotes presenter
M.C. Reneer, Gavin Ellis*, Lee Kiefer, Alex Kiefer, A. McCool, F. Marti. “Signaling Mechanisms Involved in the Blockade of TCR Induced AKT Activity in Primary Human iTregs.” 2011 40th Autumn Immunology Conference (AIC), Chicago, IL.

M.C. Reneer*, A.C. Vélez-Ortega, A. Norris, F. Marti. “Defective TCR-dependent Activation of the AKT Pathway in Peripherally Induced Tregs.” 2010 39th Autumn Immunology Conference (AIC), Chicago, IL.


ABSTRACTS - CONTINUED


Kermicle, Mary Catherine*, Bakker, Martin. “Making Mesophorus Silica Incorporating Nanoparticles, Dendrimer, and Pinacyanol Bromide into the Pores.” INSPIRE Polymer and Biomedical Science Conference, Hattiesburg, MS.

AWARDS/HONORS
• 2010 – Travel Grant Awarded from University of Kentucky for AIC Conference
• 2010 – ASNEMGE National Scholar Award at UEGW international conference in Barcelona, Spain
• 2009 – Travel Grant Awarded from University of Kentucky for AIC Conference
• 2006 – INSPIRE Conference 2nd place in Oral Presentation

PROFESSIONAL ACTIVITIES
• Graduate Women in Science Beta Chi Chapter, Member 2011 to present.
• PGY TEAM (Physiology Teaching Education and Mentoring), Member 2010 to present
VOLUNTEER EXPERIENCE

- Mentored four graduate students and one high school student in my lab
- Career Day Participant Southern Elementary School 2012
- Science Fair Judge at Sts. Peter and Paul Catholic School 2009
- Science Fair Judge at Cassidy Elementary School 2008-2011