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ON T CELL FATE DECISIONS: RETINOL, METABOLISM AND ITREG DIFFERENTIATION

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ON T CELL FATE DECISIONS: RETINOL, METABOLISM AND ITREG
DIFFERENTIATION

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
Gavin Isaac Ellis

Lexington, Kentucky

Director: Dr. Francesc Martí
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Immunology & Molecular Genetics

Lexington, Kentucky

2013

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ABSTRACT OF DISSERTATION

ON T CELL FATE DECISIONS: RETINOL, METABOLISM AND iTREG DIFFERENTIATION

The mammalian immune system is equipped to both eliminate pathogenic microorganisms and tumors, while remaining in homeostasis with commensal species at mucosal surfaces and tolerant towards self. Suppressor regulatory T cells (Tregs) are a major sentinel of this immunological tolerance. Induced Tregs (iTregs) arise in the periphery following the integration of cues from the metabolites, cytokines, etc. which make up its milieu. Dysregulation of iTreg development, function or homing underlies the etiology of many autoimmune diseases and immunopathologies. The amelioration or prevention of multiple murine disease models by boosting Treg cell numbers foreshadows clinical efficacy of iTreg therapy, but an incomplete understanding of Treg development has thus far prevented successful translation. Therefore, we considered the basic biology of T cell fate decision making from two unique, but integrated angles. First, we show that the stimulation of PPAR γ in human T cells upregulates RDH10, a molecule which catalyzes the rate limiting step in the oxidation of retinol to transcriptionally active all-trans retinoic acid (ATRA), a positive regulator of iTreg development. This functionally intact pathway endows T cells the ability to autonomously sense and respond to retinoid signals present during Treg development and at tissue sites. Next, we asked questions about how T cells sense nutrient and oxygen availability as they differentiate. Tregs lacking the serine/threonine kinase PINK1 have limited activation-induced phosphorylation of Akt and oxidative phosphorylation rates, and reduced suppressor function. Notably, the uncoupling of iTreg function from normal FoxP3 expression reinforces the recent hypothesis that the PI3K/Akt/mTORC1 axis and metabolic checkpoints are decisive players in the acquisition of suppressor activity. Ultimately, the studies described herein converge on Akt and metabolism, and contribute to our understanding of how T cells integrate diverse signals present during fate determinism, provoking future Treg based therapeutics.

Keywords: Tregs, PPAR γ , IBD, Metabolism, Akt

Gavin Isaac Ellis

Date

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DIFFERENTIATION

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Dedicated to
Eamonn Thomas McGarvey (1981-2006)

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As I sit down to acknowledge the people who were vital in my arriving to this point, I can't help but notice the similarity in the "fate decisions" I have made in my own life and those which I describe in the main body of this text-evaluating the external signals in my own microenvironment, and becoming more mature, differentiated, specialized as I go along. I started out completely undifferentiated, in the "thymus" of my suburban Philadelphia home. At this nascent stage, my parents were my main influences. I will always be grateful to Barnett Ellis for being the anti-repressor in my life. He never pushed me in one direction or another, but he always let me know that I could be whatever I want to be, and to make sure I always had the options to choose my own path. On the other hand, Barbara Ellis was my promoter. She was always the influence that pushed me towards science, technology and medicine; there had to be a reason Morris Holender (whom I would also like to acknowledge), always called 5 year old Gavin, "Doogs." Of course, neither of my parents would be complete without heterodimerization with Janet Ellis and Edward Wermuth, respectively, who helped my mom and dad raise me. I would be remiss without mentioning my first roommate (or womb-mate), my dizygotic twin and my best friend Joshua Ellis. I am grateful that I've been able to share my entire life so far with him, going through the same experiences at the same time, and could not be happier for him that he's found his new best friend, my new sister-in-law Lauren Bilardo. I don't think Josh was ever aware of what was going on in the lab, and that was partially by design on my part. Anytime I was feeling overwhelmed, exasperated or frustrated in the lab, Josh was there to fill me in on some completely false transfer rumor, momentarily and vitally allowing me a mental outlet from my laboratory bubble.

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TABLE OF CONTENTS

| | |
|---|-----|
| Acknowledgements..... | iii |
| List of Figures..... | ix |
| Chapter 1: Introduction | |
| 1.1 The “problem” of immunity..... | 1 |
| 1.2 Immunological tolerance: the solution to the problem | 2 |
| 1.3 Treg control of intestinal homeostasis | 7 |
| 1.4 Signaling an iTreg response | |
| 1.4.1 The PI3K/Akt/mTOR axis: a hub of T cell fate determinism..... | 11 |
| 1.4.2 The minimal iTreg inducers: TGF- β and IL-2..... | 13 |
| 1.5 Summary..... | 14 |
| Chapter 2: Materials and Methods | |
| 2.1 Generation of Induced Regulatory T Cells from Primary Human Naïve and Memory T Cells | |
| 2.1.1 Isolation CD4+ CD25- T cells from human blood donors | 19 |
| 2.1.2 Generation of iTregs and Th17 cells from CD4+ CD25- precursors..... | 20 |
| 2.1.3 T cell suppressor assay..... | 20 |
| 2.2 Flow cytometry | 21 |
| 2.3 Microarray..... | 22 |
| 2.4 Western Blot | 22 |
| 2.5 qPCR..... | 24 |
| 2.6 Immunoprecipitation..... | 24 |
| 2.7 Electrophoretic mobility shift assay (EMSA)..... | 24 |
| 2.8 Mice | 25 |
| 2.9 <i>in vitro</i> T cell activation..... | 25 |
| 2.10 Extracellular flux analysis..... | 26 |
| 2.11 Statistical analysis..... | 27 |
| Chapter 3: Human iTreg development is enhanced by the T cell autonomous all-trans retinoic acid synthesis pathway mediated by PPAR γ | |
| 3.1 Introduction..... | 31 |
| 3.2 Results | |
| 3.2.1 PPAR γ is necessary for optimal human Treg development..... | 34 |
| 3.2.2 Retinol enhances iTreg generation in human CD4+ CD25- T cells downstream of PPAR γ | 35 |
| 3.2.3 PPAR γ increases transcription of RDH10 via direct binding..... | 36 |
| 3.3 Discussion | 37 |
| Chapter 4: Mitochondrial and cytosolic roles of PINK1 shape iTreg development and function | |
| 4.1 Introduction..... | 59 |

| | |
|---|-----|
| 4.2 Results | |
| 4.2.1 Absence of PINK1 attenuates Akt activation in response to TCR stimulation..... | 64 |
| 4.2.2 PINK1 is required for full early activation and proliferation of T cells | 64 |
| 4.2.3 Induced regulatory T cell development and function requires PINK1 | 67 |
| 4.3 Discussion | 67 |
| Chapter 5: Discussion | |
| 5.1 The activities of PPAR γ /RDH10 and PINK1 are integrated through Akt/mTOR and metabolism | 87 |
| 5.2 Overall significance | 89 |
| 5.3 Conclusion | 91 |
| References | 94 |
| Vita..... | 110 |

LIST OF FIGURES

| | |
|--|----|
| Figure 1.1, T cell fate decisions..... | 16 |
| Figure 1.2, Mechanisms of iTreg immunosuppression..... | 17 |
| Figure 1.3, TCR, CD28 and IL-2 receptors activate the PI3K/Akt/mTOR axis..... | 18 |
| Figure 2.1, Generation of iTregs from human peripheral blood donors..... | 28 |
| Figure 2.2, Representative plot of iTreg differentiation from CD25- human donor cells..... | 29 |
| Figure 2.3, The iTreg subset (CD25 ^{hi} CD45RA-) has demonstrable suppressor activity | 30 |
| Figure 3.1, PPAR γ is necessary for optimal human iTreg development..... | 45 |
| Figure 3.2, PPAR γ controls the endogenous retinoic acid metabolism pathway in human iTregs through RDH10..... | 48 |
| Figure 3.3, PPAR γ directly binds to RDH10..... | 55 |
| Figure 3.4, Chapter 3 model..... | 58 |
| Figure 4.1, Schematic of the electron transport chain and oxidative phosphorylation..... | 74 |
| Figure 4.2, TCR crosslinking of peripheral CD4 ⁺ T cells requires PINK1 for optimal Akt activation..... | 75 |
| Figure 4.3, Flow cytometry analysis of thymus cells from wild type and <i>PINK1</i> ^{-/-} mice | 76 |
| Figure 4.4, Reduced activation of CD4 ⁺ T cells in <i>PINK1</i> ^{-/-} mice..... | 77 |
| Figure 4.5 Extracellular flux analysis of activated CD4 ⁺ T cells reveals metabolic deficiencies downstream of IL-2 pathway..... | 79 |
| Figure 4.6, Reduced suppressor activity in <i>PINK1</i> ^{-/-} iTreg cells..... | 81 |
| Figure 4.7, Chapter 4 model..... | 86 |

Chapter 1: Introduction

1.1 The “problem” of immunity

There was a brief moment in history after the origin of life, but before the first cell division, where the first single celled organism existed in complete singularity and immunity didn't exist; it didn't need to. Reproduction begot speciation, speciation created competition and competition necessitated the development of a defense system which would become immunity. The proto-immune system acted to degrade foreign nucleic acids, followed by the development of innate immunity in eukaryotes – quick, but only able to recognize patterns of molecules commonly found on pathogens, and without the ability to finger repeat offenders. Ever-increasing organismal complexity and the evolution of novel and diverse forms of invasion commanded the development of the adaptive immune system, originally seen in the gnathostome superclass of jawed vertebrates. The ideal immune system would quash invaders before having a chance to expand in number, yet retain adequate specificity and to be able to respond to an unlimited array of antigens.

Adaptive immunity is bifurcated into cell-mediated and humoral immunity, the T and B lymphocytes, respectively. T cells have diverse functions within the immune system, but generally kill infected or aberrant cells, activate complementary arms of the immune system including antibody secretion from B cells or suppress inappropriate pro-inflammatory responses. A T cell's specificity is encoded within its T cell receptor (TCR), which is uniquely and randomly rearranged during T cell development. The crosslinking of the TCR by cognate peptide presented by antigen presenting cells (APCs) drives the activation and expansion process of clonal expansion. TCR gene

rearrangement during T cell development has the capacity to generate an unlimited number of dissimilar TCRs. Herein lies the problem of immunity: to ensure no pathogen goes undetected, the process of TCR diversification must produce T cells which recognize self, benign or beneficial antigens, resulting in autoimmunity, allergy and immunopathogenesis. Immunological tolerance became the solution.

1.2 Immunological tolerance: the solution to the problem

After egress from the bone marrow, pluripotent hematopoietic stem cells populate the thymus, where Notch1 signaling from stromal cells commits them to the T cell lineage, and incites TCR rearrangement and expression of both the CD4 and CD8 co-receptors. Education of T cells to ensure tolerance occurs almost immediately. These “double positive” (DP) cells interact with self-peptide:self-major histocompatibility complex (MHC) in the thymic cortex and undergo positive selection by integrating TCR and co-receptor signals. DP cells that cannot bind to and signal through CD4 or CD8 will undergo apoptotic death by neglect, whereas successful signaling provides a proliferation signal, upregulates TCR expression and downregulates expression of the unused co-receptor, leaving either a single positive (SP) CD4⁺ or CD8⁺ lymphocyte depending on the class of MHC encountered; this dissertation will focus on CD4⁺ T cells. Negative selection occurs next: SP T cells interact with medullary thymic epithelial cells expressing an array of self-peptides under the control of the autoimmune regulator, Aire. Cellular fate at this juncture is determined by the strength of the signal: T cells which react too strongly to self-peptide are destined for apoptosis, as they may cause autoimmunity, intermediate binding results in the development of anti-inflammatory natural regulatory T cells (nTregs) and the remaining cells leave the thymus as mature,

naïve T cells. A full 98% of thymocytes do not survive T cell development, but the 2% that do are fully-armed cells that have the ability to recognize and respond to any protein:MHC complex that later binds faithfully. Notably, the intertwining of tolerance and T cell maturation is a testament to the commitment of the immune system to the prevention of autoimmunity, and represents the first stage at which the integration of signals helps determine T cell fate.

Upon egress from the thymus, naïve CD4⁺ T cells are on constant lookout for cognate antigen presented by APCs in the context of MHC class II molecules. Since most T cells with self-reactive TCRs are deleted during the negative selection phase of thymic development, the majority of T cells search for foreign antigens. The odds of a productive T cell:APC event in peripheral blood is quite low, so naïve T cells traffic to leukocyte-rich secondary lymphoid organs such as lymph nodes, spleen and mucosal associated lymphoid tissue, including Peyer's patches in the gut. After entering one such organ, a T cell will interact with thousands of dendritic cells (DCs) per day, sampling the buffet of peptide:MHC complexes on the surface of the APCs. The vast majority of these interactions will be brief – only lasting seconds to minutes on average – before a T cell moves on to the next APC, ultimately ending up back in the blood if nothing piques its interest. On rare occasions, a T cell will suitably bind to an APC with enough affinity that TCR signaling will stabilize the interaction. Additional co-stimulatory signals are provided by molecules like CD28, which initiate the cell cycle, interleukin-2 (IL-2) secretion and expression of the α -chain of the IL-2 receptor, CD25. TCR activation of naïve T cells in the absence of CD28 co-stimulation induces T cell anergy, and is another mechanism of tolerance. It is during TCR stimulation that activation, clonal expansion

and T cell effector fate decision making occurs based on integration of the signals received from a cell's local microenvironment.

Though organismal complexity has increased via an evolutionary random walk away from the simplest forms of life, bacteria, viruses and other single-celled microorganisms are to this day the modal organisms on this planet, and with this abundance comes diversity¹. Organisms have evolved to infect, grow, persist and reproduce in every available tissue niche in the human body, and the most successful of pathogens subvert, depress, hoodwink or misdirect the human immune system: HIV hides out in CD4⁺ T cells and integrates its genetic information into the host genome, *Trypanosoma brucei* drastically changes its variant surface glycoprotein coat, etc. Therefore, the immune system must tailor its response to fit the pathogen encountered and the body site in which it is found. Part of this adaptation involves the orchestration of distinct CD4⁺ T cell subsets. A T cell undergoing activation acquires its homing specificity and cytokine secretion profile depending on the signals it receives from the environment. Though initially thought to be distinct and immutable, CD4⁺ T cell subsets *in vivo* are actually variegated and plastic – CD4⁺ T cells can exhibit characteristics of multiple subsets and can re-differentiate into a new subset based on subsequent microenvironmental signals². In general, pro-inflammatory CD4⁺ T cells are classified into three subsets: Th1, Th2 and Th17. The cytokine signals, master transcription factors and signature cytokine secretion profiles of each of the major subsets are listed in Figure 1.1, in addition to their primary functions. Improper T helper responses can not only be ineffective, but also pathogenic. For example, initiating a Th2 response against *Mycobacterium leprae* will allow it to persist inside macrophages and cause lepromatous

leprosy³, and Th1-mediated inflammation in the brain could cause damage due to swelling within the enclosed cranium and induced cell death of a non-replicating cell type.

In contrast to the pro-inflammatory response, some T cells become induced Tregs (iTregs), an anti-inflammatory subset of CD4⁺ T cells instructed to develop by the coordinated action of the cytokines transforming growth factor- β (TGF- β) and IL-2. Like their nTreg counterparts, iTregs develop following the induction of its master transcriptional regulator, FoxP3⁴, and in humans, can be identified by their high expression of CD25⁵, CTLA-4⁶, GITR⁷ and low expression of CD127⁸. Though nTregs and iTregs have yet to be assigned distinct physiological roles, Helios is a zinc-finger protein expressed by nTregs, but not by iTregs⁹, and the conserved noncoding sequence 1 (CNS1) region of *Foxp3* contains binding sites essential for iTreg development, but dispensable for nTregs¹⁰. Contemporary opinion supposes that these etiological differences between nTregs and iTregs contribute to their distinct TCR repertoires, and assigns separate, but still overlapping regulatory roles in the control of inflammation¹¹; nTregs were hypothesized to tolerize towards self antigens¹², while iTregs filled the non-self niche¹³. However, these roles are still being experimentally defined, and single-cell sequencing indicates that the majority of Tregs are thymus derived, including those in the colon, which is replete with non-self antigens¹⁴. Though tolerance via nTreg activity may be sufficient in young individuals with large thymi, thymic involution decreases output of all T cells including nTregs, suggesting a continuing role for iTregs later in life¹⁵, and ablation of iTregs results in Th2-type immunopathogenesis at mucosal surfaces¹⁶.

FoxP3 itself binds to DNA at its consensus sequence¹⁷ where it acts as a transcriptional repressor. Cytokine genes are a major target of FoxP3, which reduces the secretion of IL-4, interferon- γ (IFN- γ) and the IL-2 which supports its development and stability¹⁸. Though it holds true in mice, the discovery of FoxP3⁻ Tregs and FoxP3⁺ cells lacking suppressor function^{19,20} has shown FoxP3 to be neither necessary nor sufficient for human iTreg development. Tregs suppress through several cell contact dependent and independent mechanisms (Figure 1.2), and results in multiple anti-inflammatory outcomes including reduced proliferation, differentiation and cytokine secretion of bystander immune cells. It is a key feature of Tregs that they are activated in an antigen-specific manner, but suppress non-specifically. In addition, Tregs can impart infectious tolerance by manipulating the T cell microenvironment to such that fosters immunosuppression²¹; adoptive transfer of congenically marked Tregs to non-obese diabetic mice prevented pathology despite their transient persistence. Instead, newly generated, marker-negative Tregs assumed the responsibility of reinforcing tolerance and preventing a diabetic state²².

The contribution of FoxP3 and Tregs towards tolerance was recognized upon the creation of hemizygous “scurfy” mice carrying a mutated *FoxP3* lacking its forkhead domain, which succumb to a lymphoproliferative disease marked by multi-system autoimmunity²³. Concordant symptoms were seen in humans diagnosed with immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX), leading to the identification of multiple loss-of-function mutations in FoxP3 as the causative agent of disease²⁴. Since this initial characterization, several autoimmune diseases have

likewise been at least partially attributed to dysregulation of Treg-mediated immunosuppression^{22,25–28}.

1.3 Treg control of intestinal homeostasis

Nowhere is tolerance more relevant than in the over 400 square meters of gastrointestinal tract that houses 10^{14} microbial residents at a density of up to 10^{12} organisms per cubic centimeter. Because food and nutrients must be absorbed through the gut wall, the epithelial cell layer separating the underlying immune cells of the lamina propria from the gut lumen is only a single cell thick, seemingly setting the stage for uncontrolled inflammation directed against enteric bacteria. However, there exists a panoply of mechanisms which ensure peaceful co-habitation between the body and the microbiota, which is clearly illustrated by the number and diversity of mouse models that cause intestinal inflammation. Administration of dextran sodium sulfate (DSS) to drinking water abrades the epithelial cell layer, causing an innate form of colitis that occurs even in *SCID* mice lacking adaptive immune cells. In the T cell transfer model of colitis, the transfer of Treg-depleted $CD4^+$ T cells into an immunocompromised host causes intestinal inflammation²⁹. Colitis can also be initiated by particularly invasive bacterial species³⁰ or genetic ablation of vital anti-inflammatory molecules, as in the case of the IL-10 knockout mouse³¹. For many of these models, mucosal inflammation only occurs when mice are housed inside a conventional animal facility that is permissive of the establishment of a gut microbiota³¹ and can be ameliorated by antibiotic treatment³², exhibiting specificity of the response.

In addition, the human gut experiences an estimated 10-15 kilograms of non-self proteins per year from dietary sources. Loss of tolerance towards these antigens is the basis of many food allergies and pathologies like celiac disease, an inappropriate inflammatory reaction against gluten. Aside from the symptoms of abdominal pain and cramping, intestinal inflammation reduces the ability to absorb fat soluble vitamins, calcium or other nutrients from food sources. Immunological homeostasis with ingested antigens is established by mechanisms of oral tolerance, and is not restricted to food antigens. CD4⁺ T cells from mice fed myelin basic protein can ameliorate experimental autoimmune encephalomyelitis directed against this antigen in part by TGF- β production³³, and the ingestion of poison ivy has been a strategy long-since employed by Native Americans to combat urushiol-induced contact dermatitis³⁴. In a model of ovalbumin feeding, antigen specific FoxP3⁺ regulatory T cells were efficiently generated in the mesenteric lymph nodes (MLN), lamina propria and Peyer's patches following oral administration³⁵.

The lack of sterility found at mucosal surfaces necessitated a specialized immune system that reacts differently than that of the rest of the body. Though the immune system must tolerate commensal and food antigens, it must be able to identify and eliminate enteric pathogens, and permit extra-mucosal immunity against commensals to persist in the case of bacterial invasion. Dynamic, low level inflammation keeps bacterial symbionts in check via tightly regulated homeostasis between lymphocytes including activated and regulatory T cells – non-activated T cells are not often found in the intestine. To achieve this goal, three-quarters of all lymphocytes are located within the mucosal immune system, and mucosal B cells produce between 3 and 5 grams of

immunoglobulin A per day. Within the intestine, T cell activation occurs in the gut-associated lymphoid tissue, organized lymphoid regions including Peyer's patches which drain via lymphatics to the MLN. Dendritic cells in the lamina propria extend their processes between epithelial cell tight junctions³⁶ to sample luminal antigens, while those in Peyer's patches have direct access through microfold cells (M cells). Upon antigen uptake, DCs alter their homing properties and traffic to the MLN where they present antigen to gut T cells along with instruction for activated T cells to remain within the mucosal immune system.

In humans, the loss of peripheral tolerance towards commensal bacterial antigens in the gut leads to the development of Crohn's disease (CD) or ulcerative colitis, disorders collectively known as inflammatory bowel diseases (IBDs). Although both morbidities are often difficult to separate diagnostically, each has its own hallmarks. Crohn's disease patients develop non-caseating granulomatous inflammation which can manifest anywhere in the GI tract, but largely develops in the colon or ileum as discontinuous "skip lesions". Inflammation is usually transmural and commonly leads to fibrotic strictures or fistulae. Crohn's has classically been considered a Th1 mediated disease due to expression of the Th1 signature transcription factor, T-bet and increased secretion of IFN- γ , IL-2, IL-18 and IL-12. However, this paradigm is now in flux, as many of the disease qualities attributed to IL-12 must now be re-characterized because of the historical use of antibodies which did not differentiate between IL-12 and the Th17-produced IL-23. IL23p19 knockout, but not IL12p35 knockout mice were protected from disease in a transfer model of colitis³⁷, and monoclonal antibodies directed against

IL23p19 ameliorate symptoms in a *Helicobacter hepaticus* model of intestinal inflammation³⁸.

On the other hand, ulcerative colitis is a continuous, Th2 mediated inflammatory disease manifesting exclusively in the colon and marked by increased levels of TGF- β and IL-5. Ulceration of the intestinal wall in colitis is not as deep as is seen in Crohn's disease. Although ulcerative colitis can be completely cured by removal of the inflamed gut region, it is only recommended for patients with excessive blood loss, a high risk of colon cancer or gastrointestinal perforation³⁹. Despite the number of differences between the two pathologies, 5-15% of IBDs cannot be classified as Crohn's or ulcerative colitis and are termed "indeterminate colitis."⁴⁰ Concordantly, several therapies such as monoclonal antibody neutralization of the Th1 cytokine tumor necrosis factor- α (TNF- α) have shown efficacy in both Crohn's disease and ulcerative colitis, demonstrating the similarity between pathologies⁴¹.

Notably, no mouse model of intestinal inflammation fully recapitulates the clinical features of either Crohn's disease or ulcerative colitis, underscoring the diversity of disease in IBD patients due to the equal contributions of genetics, the environment and the microbiota, and making targeted therapeutics a less viable option for disease amelioration versus a more general approach. Several mouse models of autoimmune disease provide proof-of-principle for iTreg based therapies including IBD⁴², arthritis²⁷ and autoimmune diabetes⁴³. Yet, several key blockades relating to differences in murine and human T cell biology must be resolved before this approach appears in the clinic. While FoxP3 is unique to Tregs in wild type mice, human FoxP3 expression can be found in activated cells without suppressor activity²⁰ and can be lost by Tregs that subsequently

gain effector function². Moreover, the large titers of pooled Tregs needed to enact a therapeutic benefit in mouse models of autoimmunity suggest the same will be true in human disease, which will require adequate expansion from FoxP3⁻ precursors. Because of our inability to identify pure populations of human iTregs, the transfer of a sorted FoxP3⁺ population of cells would be contaminated by effector cells, exacerbating symptoms. Therefore, the identification of molecular and genetic signals involved in the development and expansion of stable, uniquely identifiable Tregs is an active area of research.

1.4 Signaling an iTreg response

1.4.1 The PI3K/Akt/mTOR axis: a hub of T cell fate determinism

The interaction of a T cell with its appropriate antigen on the surface of an APC is the first step in the activation of a CD4⁺ T cell. As part of this process, TCR interaction with peptide:MHC complexes, CD28 binding to B7 molecules and signaling of IL-2 through its receptor (IL-2R) all activate the phosphoinositide 3-kinase (PI3K) signaling pathway within seconds, modifying cellular processes to support clonal expansion and differentiation. Activated PI3K phosphorylates phosphatidylinositol (4,5)-diphosphate (PI-4,5P₂), yielding the PI-3,4,5P₃ needed to recruit pleckstrin homology (PH) domain containing proteins to the inner leaflet of the plasma membrane including the serine/threonine kinase Akt, a protein at the hub of several pathways controlling host proliferation, growth, apoptosis, differentiation and metabolism^{44,45}.

Though it was originally recognized for its role in viral oncogenesis⁴⁶, Figure 1.3 shows the positioning of Akt downstream of receptor tyrosine kinases including the insulin receptor, TCR and CD28 co-stimulatory receptor. Each of the three isoforms of

Akt (Akt1, Akt2 and Akt3) is organized into three functional domains. The N-terminal PH domain endows Akt the ability to bind PI-3,4,5P₃, and to a lesser extent PI-3,4P₂, embedded in the cytosolic side of a cell's plasma membrane. Its central kinase domain preferentially phosphorylates the protein consensus sequence RXXXX(S/T), which is controlled by the molecule's C-terminal regulatory domain.

Activation of Akt begins with a conformational change to reveal two phosphorylation sites within the regulatory domain. Subsequent phosphorylation of threonine 308 (T308) by phosphoinositide-dependent kinase-1 (PDK1) and serine 473 (S473) by a yet unknown kinase then activates the kinase activity of Akt. Though Akt regulates mammalian target of rapamycin (mTOR) via phosphorylation, evidence exists that mTOR complex 2 (mTORC2), of which mTOR is a member, could be the identity of one of the kinases responsible for S473 phosphorylation in a reciprocal type of regulatory relationship⁴⁷. mTOR, a serine/threonine kinase in its own right, acts as a component of one of two multi-protein mTORCs. When mTOR conglomerates with several proteins including raptor and Rheb, mTORC1 has distinct qualities from mTORC2 that includes rictor, including sensitivity to inhibition by the small molecule rapamycin. Downstream of Akt, mTORC1 promotes the protein synthesis needed for cell growth and proliferation by phosphorylating p70 ribosomal S6 kinase 1 (S6K1) to facilitate transcription⁴⁸ and eukaryotic initiation factor 4E (eIF4E) to enable cap-dependent translation⁴⁹.

Downstream effects of the PI3K/Akt/mTOR have a pronounced role during T cell fate decision making. In mice with a T cell specific deletion of the mTOR gene *Frap1*, proliferation was attenuated following CD3/CD28 stimulation, and neither Th1 nor Th2 nor Th17 polarizing conditions were able to impart the ability to produce IFN- γ , IL-4 or

IL-17, respectively. Instead, stimulated *Frap1*^{-/-} T cells express FoxP3 and assume regulatory T cell function, even in response to the Th1-polarizing *Vaccinia* virus *in vivo*⁵⁰. In this way, iTregs can be seen as the default differentiation pathway, away from which mTOR is able to cause deviation. Alternatively, forced constitutive activation of Akt antagonizes FoxP3 development⁵¹.

The type of mTORC activated following TCR stimulation directs effector cell subset differentiation. In *Rheb*^{-/-} mice which lack mTORC1 activity but have sufficient mTORC2, *Vaccinia* instead provokes the generation of IL-4⁺ Th2 T cells, and fewer IL-17⁺ cells are found in Peyer's patches. Conversely, Th2 cells rely on mTORC2; *Rictor*^{-/-} T cells develop into Th1 and Th17 cells, but not Th2 cells. Although the exact mechanisms by which the PI3K/Akt/mTOR axis works have not yet been elucidated, cytokine receptor associated signal transducer and activator of transcription (STAT) proteins have reduced phosphorylation without mTOR expression⁵².

1.4.2 The minimal iTreg inducers: TGF-β and IL-2

Our lab⁵³ and others have differentiated human iTregs upon culture of stimulated CD4⁺ FoxP3⁻ T cells with TGF-β and IL-2. TGF-β is a cytokine with pleiotropic effects that can vary or even oppose each other based on cell type, activation state or host species⁵⁴. Signal transduction begins when TGF-β binds to receptor type II, allowing it to recruit and phosphorylate receptor type I (TGF-βR1). TGF-βR1 is a kinase with specificity for the receptor-activated transcription factors Smad2 and Smad3. Upon phosphorylation, Smad2 and Smad3 form a Smad complex via association with Smad4, and translocate to the nucleus to bind DNA and modulate transcription. Additionally, TGF-β exhibits Smad-independent signaling character, including the activation of Akt⁵⁵.

In T cells, TGF- β is a differentiation factor for both Th17 and Treg cells, depending on its integration with other cytokine signals. TGF- β activation with concomitant IL-6 stimulation results in Th17 differentiation, while TGF- β plus IL-2 induces Treg development⁵⁶. For Tregs specifically, Smad3 binds with the TCR-inducible transcription factor nuclear factor of activated T cells (NFAT) to the CNS1 region of *Foxp3* to induce its expression^{10,57}, and *TGFB*^{-/-} mice succumb to multi-organ autoimmunity⁵⁸. By secretion of TGF- β themselves, iTregs are able to engage in infectious tolerance.

IL-2 is a cytokine that is a T cell growth and differentiation factor, but can also induce activation-induced cell death⁵⁹. The IL-2R exists as a heterodimer of one molecule of its β chain (CD122) in complex with one common γ chain (CD132) and is able to signal in response to high levels of IL-2. Upon expression of the α chain (CD25) in activated T cells and Tregs and subsequent binding of $\alpha\beta$ to IL-2, a new high-affinity heterotrimer forms. Janus activated kinase 3 (JAK3) associated with the common γ chain and JAK1 bound to CD122 phosphorylate the β and γ chains of the IL-2R and each other to allow assembly of a complex that results in activation of STAT5. Among other roles, STAT5 binding to the CNS2 region of *Foxp3* drives Treg development in concert with TGF- β ⁶⁰. The role of IL-2 in tolerance is such that *IL2*^{-/-} and *STAT5*^{-/-} mice develop fatal autoimmunity with IBD-like symptoms marked by a massive increase in the infiltration of activated, proliferating T cells^{61,62}, which can be ameliorated by Treg transfer^{63,64}.

1.5 Summary

In this dissertation, we describe our work profiling the ability of T cells to sense and respond to extracellular signals during T cell fate decision making en route to iTreg differentiation with both mouse and human systems. This multi-species approach will

elucidate mechanisms of iTreg differentiation and function to improve our ability to design therapeutics for IBDs and other immunopathologies. In chapter 3, we describe for the first time the human T cell endogenous retinol metabolism pathway via the induction of RDH10 downstream of PPAR γ , and its pro-iTreg character. Chapter 4 contains the first phenotypic description of T cells from PINK1 knockout mice, where FoxP3 expression is uncoupled from suppressor function. Ultimately, both of these approaches converge on the PI3K/Akt/mTOR pathway and cellular metabolism, which we posit is the crux of T cell fate determinism and allows T cells to be masters of signal integration.

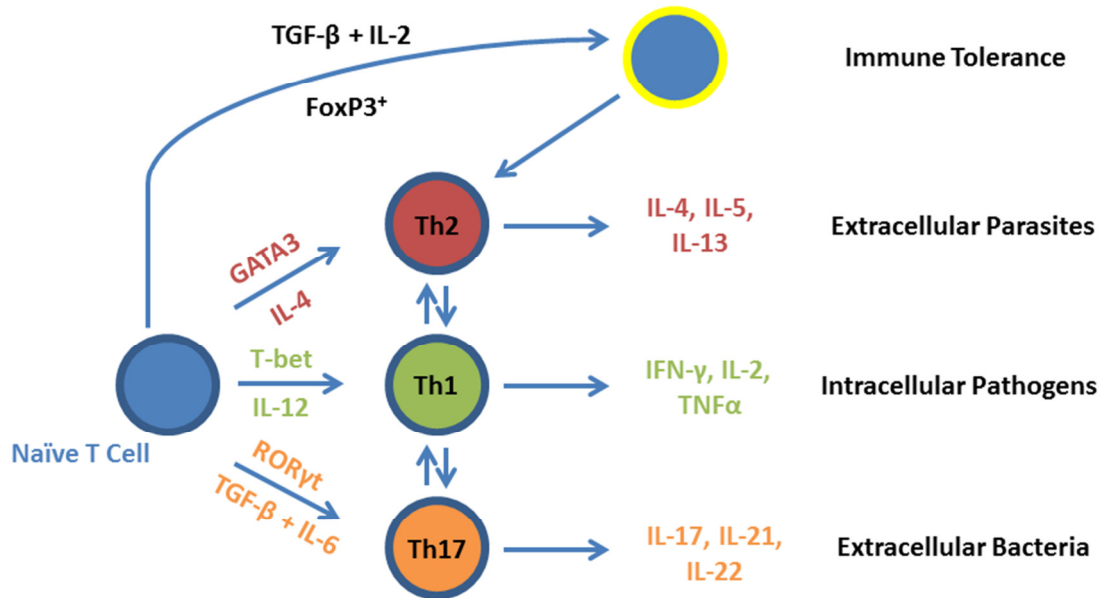


Figure 1.1 T cell fate decisions. Naïve CD4⁺ T cells integrate signals from their extracellular environment to determine their effector profile. Stimulation in the presence of IL-12 induces master transcriptional regulator T-bet, which controls the Th1 response of IFN-γ, IL-2 and TNF-α to defeat intracellular pathogens and viruses. IL-4 encountered during activation causes a Th2 program: GATA-3 prompts the secretion of IL-4, IL-5 and IL-13 to combat extracellular pathogens. RORγT directs the Th17 response after TGF-β + IL-6 exposure towards the secretion of IL-17, IL-21 and IL-22 aimed at clearance of extracellular bacteria. Alternatively, a suppressor response can be initiated by TGF-β and IL-2 through FoxP3. These designations describe T cell responses in response to pathogens in general; individual T cells can exhibit mixed character between T helper subsets and have the ability to switch from pro- to anti-inflammatory. For a more complete review of CD4⁺ T cell subset differentiation and function, including that of follicular helper T cells, Th9 cells, etc., readers are referred elsewhere⁶⁵.

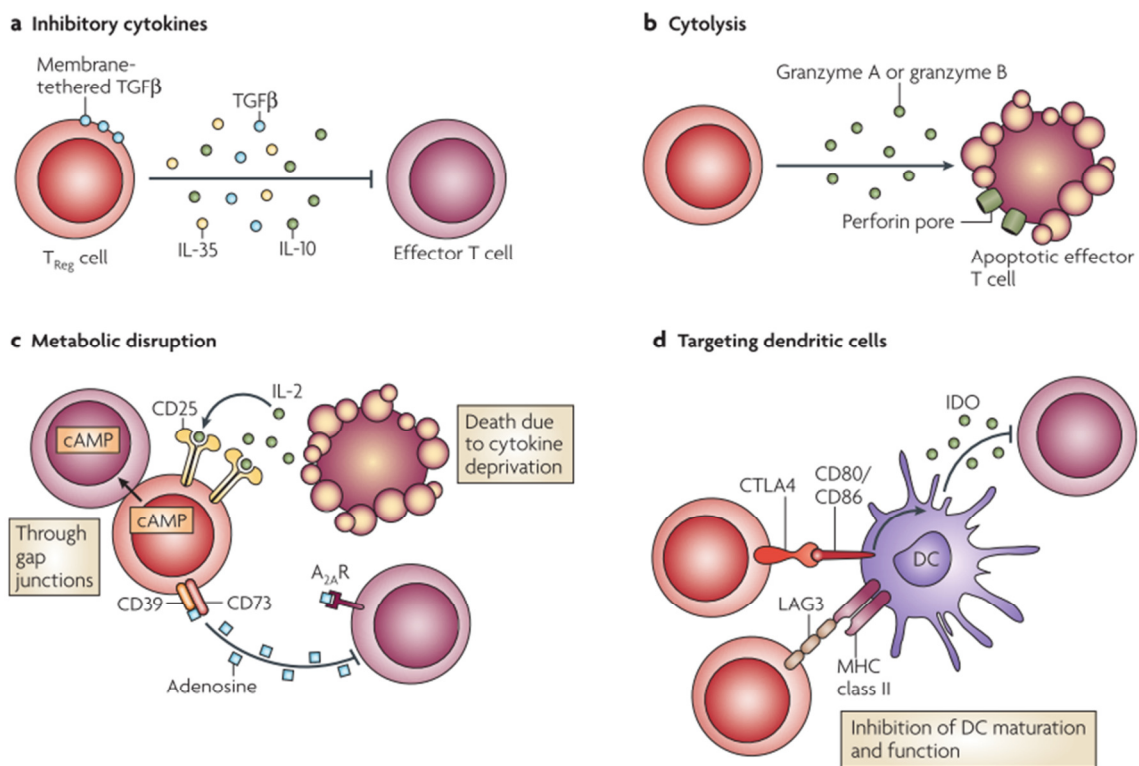


Figure 1.2 Mechanisms of iTreg immunosuppression. iTregs have multiple means of cell contact dependent and independent means of anti-inflammatory activity. Notably, many contemporary studies examine the activity of iTregs solely via their ability to suppress bystander T cell proliferation. Figure reproduced with permission from (66).

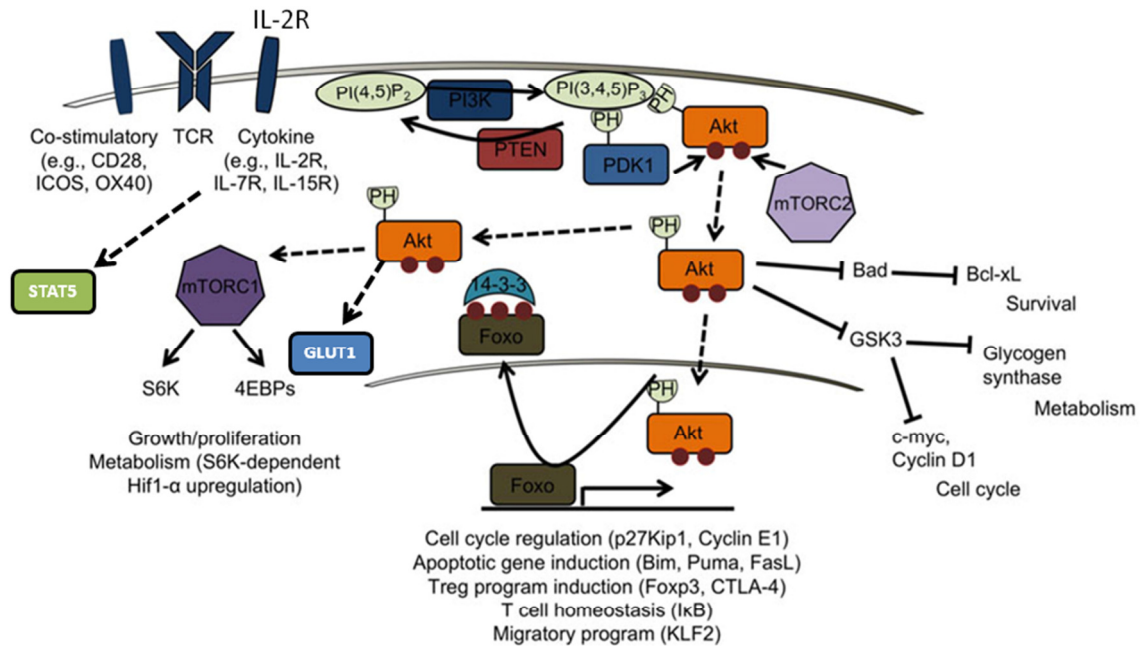


Figure 1.3 TCR, CD28 and IL-2 receptors activate the PI3K/Akt/mTOR axis. Upon T cell activation, PI3K creates a docking site for Akt at the cell membrane where it can be phosphorylated by PDK1 and mTORC2. Activated Akt binds many downstream proteins including mTORC1. Coincidentally, Akt was first cloned at the Fox Chase Cancer Center, 1.7 miles from the suburban Philadelphia house where the author lived as a 5 year old at the time⁶⁷. Figure modified from (⁶⁸), reproduced here under the terms of the Creative Commons Attribution License.

Chapter 2: Materials and Methods

2.1 Generation of Induced Regulatory T Cells from Primary Human Naïve and Memory T Cells

In response to the need for a more thorough understanding of the molecular events involved in human iTreg cell fate decision making, our lab developed a method to generate iTregs (CD25^{hi} CD45RA⁻) from human peripheral blood donors. Notably, we designed this technique to utilize sub-optimal conditions, so effector T cells (CD25^{mid} CD45RA⁻) are simultaneously generated, and memory (CD25⁻ CD45RA⁺) and naïve (CD25⁻ CD45RA⁻) T cells persist, allowing genetic comparisons to be made between subsets within an individual donor. Figure 2.1 shows a graphical schematic of this process.

2.1.1 Isolation CD4⁺ CD25⁻ T cells from human blood donors

Human donations of packed granulocytes were first diluted with phosphate buffered saline (PBS) at a ratio of 1:5, before overlaying on Lymphoprep separation medium (Axis-Shield) in 50 mL conical tubes. Tubes spun for 30 minutes at 500 x g with no brake at room temperature, leaving a sharp, buff colored layer of peripheral blood mononuclear cells (PBMCs) to transfer by pipette into a fresh tube. PBMCs were washed, and then subject to EasySep magnetic negative selection (Stem Cell Technologies) as per manufacturer's instructions. The EasySep CD4⁺ T cell kit excludes cells expressing CD8, CD14, CD16, CD19, CD20, CD36, CD56, CD66b, CD123 or TCR γ/δ via Tetrameric Antibody Complexes. CD4⁺ CD25⁻ T cell purity following execution of kit is routinely >95% and results in cells unattached to any antibodies which could interfere with downstream applications.

2.1.2 Generation of iTregs and Th17 cells from CD4⁺ CD25⁻ precursors

CD4⁺ CD25⁻ precursors fresh out of magnetic selection were resuspended in “iTreg medium” at a concentration of 2 x 10⁶ cells / mL: RPMI-1640 pre-supplemented with Glutamax-I (2.06 mM, Gibco) and HEPES buffer (25 mM, Gibco) plus fetal bovine serum (10%, Gibco), penicillin (100 U/mL, Gibco), streptomycin (100 µg/mL, Gibco), β-mercaptoethanol (50 µM, Sigma-Aldrich), IL-2 (5 ng/mL, eBioscience) and TGF-β (2 ng/mL, eBioscience). Cell suspensions were plated in α-CD3 coated plates (1 µg / mL, Clone: OKT3, Bio X Cell) for 6 days, with half of the medium being replaced on day 3. This cell culture system allows for some versatility, as agonists and antagonists were added to the cell culture medium where indicated to probe the activation or inhibition of pathways of interest, and the input population of cells was altered to examine iTreg differentiation of pure memory or naïve T cells. To adapt this protocol for mouse cells, IL-2 was used in iTreg medium at 1 ng/mL, and mouse α-CD3 (Clone: 45-2C11, Bio X Cell) was used. Where noted, all-trans retinol (1 µM), all-trans retinal (1 µM), all-trans retinoic acid (1 µM), 9-cis retinoic acid (1 µM), RO 41-5253 (1 µM) or citral (5 µM) were included in the culture medium to influence the retinoic acid pathway, and GW9662 (1 µM) was included to block PPARγ. A representative flow cytometry dot plot can be found in Figure 2.2 showing the induction of CD25, CD45RA, CTLA-4 and FoxP3 over the course of 6 days. To generate Th17 cells, enriched CD4⁺ T cells were cultured in α-CD3 coated wells (1 µg/mL) in a medium containing TGF-β (5 ng/mL) and IL-6 (eBioscience, 10 ng/mL) for 3 days, before switching to an IL-23 containing medium (eBioscience, 10 ng/mL) for another 3 days.

2.1.3 T cell suppressor assay

As stated previously, no individual protein marker or panel of markers can definitively define a human Treg cell, and detection of FoxP3 requires permeabilization of cells, rendering them as non-viable. Therefore, we identified a CD4⁺ CD25^{hi} CD45RA^{lo} CD127⁻ cell surface phenotype as our iTreg subset and used a suppressor assay to show that these are *bona fide* iTregs. First, iTregs (CD25^{hi} CD45RA⁻), memory (CD25⁻ CD45RA⁻), effector (CD25^{mid} CD45RA⁻) and naïve (CD25⁻ CD45RA⁺) T cells were sorted with a MoFlo cell sorter (Beckman Coulter). During the sorting process, CD4⁺ T cells from a heterologous donor were isolated as in section 2.1.1 and then labeled with 5 mM carboxyfluorescein succinimidyl ester (CFSE, Invitrogen) for 3 minutes at 37°C. Next, cells were resuspended in suppression assay medium: AIM-V supplemented with penicillin (100 U/mL), streptomycin (100 µg/mL), β-mercaptoethanol (50 µM), IL-2 (5 ng/mL) and TGF-β (2 ng/mL) at various ratios of sorted cells to labeled cells, but so the total amount of cells per 96 round bottom well was 2 x 10⁵. Treg inspector suppression beads (Miltenyi Biotec) were added to the suspension at a number equal to the number of the total cells to drive proliferation by crosslinking of CD2, CD3 and CD28. After 5 days of co-culture at 37°C, CFSE levels were evaluated by flow cytometer. Figure 2.3 demonstrates that iTregs were the only subset of T cells that could suppress the proliferation, and thus the CFSE dilution of labeled cells. To adapt this procedure for use with mouse cells, Mouse T-Activator CD3/CD28 Dynabeads (Gibco) were used as a stimulus instead of iTreg inspector beads and IL-2 was used in the suppression assay medium at 1 ng/mL.

2.2 Flow cytometry

3×10^5 cells were resuspended in 100 μ L flow cytometry buffer (PBS, 0.5% bovine serum albumin, 2 mM EDTA) and stained with the following extracellular antibodies at manufacturer's recommended concentrations (eBioscience): CD4, CD25, CD44, CD62L and CD45RA. For detection of intracellular proteins, cells were fixed and permeabilized as per manufacturer's instructions with the FoxP3 / Transcription Factor Staining Buffer Set (eBioscience) with 2% normal rat serum to block non-specific staining, before staining with antibodies directed against CTLA-4, ROR γ T or FoxP3. Since GLUT1 and p-Akt-473 antibodies (Santa Cruz Biotechnology Inc.) were not fluorochrome conjugated, incubation with labeled secondary anti-IgG antibody (Invitrogen) facilitated their detection. For intracellular cytokine detection, T cells were cultured simultaneously with PMA (50 ng/mL), ionomycin (500 ng/mL) and GolgiStop (BD Biosciences, 4 μ L/6 mL medium) for 5 hours at 37°C before staining with the BD Cytofix/Cytoperm Fixation/Permeabilization kit and antibodies detecting IL-2 or IL-17 (eBioscience). Flow cytometry without sorting was performed on an LSRII cytometer (BD Biosciences) and analyzed with FlowJo software (Tree Star Inc.). Cell sorting was performed as extracellular staining above with the additional steps of DNaseII addition (1 μ g/mL) and a pass through a 70 μ m filter to prevent clogging of the FACS Aria cell sorter (BD Biosciences). To evaluate $\Delta\psi$ m, cells were incubated with 30 nM tetramethylrhodamine ethyl ester (TMRE, Invitrogen) for 30 minutes in RPMI at 37°C before immediate analysis.

2.3 Microarray

Total RNA was extracted from $5-7 \times 10^6$ FACS sorted CD4+ human T cells (CD25⁻, CD25^{low} or CD25^{hi} iTregs) with TRIzol (Life Technologies), from which cDNA

was immediately generated with SuperScript III First-Strand Synthesis Kit as per manufacturer's instructions (Invitrogen). With assistance from the University of Kentucky Microarray Facility, we quantified cDNA with the Affymetrix GeneChip System (Human Genome U133 Plus 2.0 Array microchip) compared to *GAPDH* as a reference control in triplicate. Results are shown normalized to values for CD25⁺ subset.

2.4 Western Blot

To perform Western blotting, cells were first lysed with cold radioimmunoprecipitation assay (RIPA) buffer: 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride and 1X Halt Protease Inhibitor Cocktail (Thermo Scientific). Next, protein lysates were mixed with 2X Laemmli buffer and boiled for 10 minutes at 90°C before being loaded in wells of a 10% or 12% SDS-PAGE gel. Gels were then run at a voltage of 100 V for 1-2 hours to separate proteins by size. Proteins were transferred to a polyvinylidene fluoride (PVDF) membrane which was then blocked for 1 hour at 37°C in 2% fetal bovine serum in TTBS buffer (50 mM Tris, 150 mM NaCl, 0.05% Tween-20). Membranes were incubated overnight at 4°C in primary antibody solution diluted as per manufacturer's instructions, washed and, when applicable, incubated at room temperature for 30 minutes in antibody solution containing secondary α -IgG antibody conjugated to horseradish peroxidase. Proteins were detected via chemiluminescence (Pierce ECL Western Blotting Substrate, Thermo Scientific) and autoradiography film. When needed, blots were stripped of antibody with stripping solution (2% sodium dodecyl sulfate, 62.5 mM Tris HCl pH 6.8 and 100mM β -mercaptoethanol) and 30 minutes of incubation at 55°C before re-probing as above.

2.5 qPCR

5×10^6 magnetically sorted CD4⁺ T cells were cultured for 24 hours in iTreg medium containing either DMSO, RSG (1 μ g) with 9-cis RA (1 μ g) or GW9662 (1 μ g) and 9-cis RA (1 μ g) on α -CD3 coated plates to stimulate or block PPAR γ , respectively. RNA was isolated from cells with the Quick-RNA MiniPrep kit (Zymo Research) per manufacturer's instructions, followed by cDNA creation from 0.5 μ g RNA with SuperScript III First Strand Synthesis System (Invitrogen). 4 μ L of cDNA was run in a 10 μ L reaction with 5 μ L PerfeCTa qPCR FastMix (Quanta Biosciences) and 0.5 μ L of a 20x pre-made primer:probe Taqman gene expression assay (Applied Biosystems). The following primers:probe mixes were used: GAPDH: Hs99999905_m1 and RDH10: Hs00416907_m1. Reaction was run on the "Quick Start" setting of a StepOnePlus RT-PCR system (Life Technologies). Data were analyzed by the $\Delta\Delta C_T$ method.

2.6 Immunoprecipitation

PPAR γ (Cell Signaling, Clone 81B8) or IgG control antibodies were conjugated to A/G PLUS agarose beads (Santa Cruz) for 30 minutes at room temperature before washing three times. Lysates were then added to beads and incubated overnight at 4°C.

2.7 Electrophoretic mobility shift assay (EMSA)

Day 6 T cell lysates were obtained by lysing cells in RIPA buffer. Detergents were removed from lysates by dialyzing proteins into dialysis buffer (10% glycerol, 1 mM EDTA, 100 μ M PMSF, 50 mM Tris pH 7.5, 0.01% Tween-20, 50 mM KCl, 1 mM DTT) with a Slide-A-Lyzer 3.5K dialysis cassette (Thermo Scientific). Each 1 μ L of the resulting lysate contained protein from ~12,000 cells. The following single stranded DNA probes were created by self-ligation with their exact complements by heating up

equal volumes of 10 mM oligonucleotide solutions in boiled water for 10 seconds and letting cool to room temperature (PPRE in bold): Wild type RDH10 5'-CTGTTTCTCCTAAAT**AGGTC**CATTAAACCTCATTA-3', Mutant RDH10 5'-CTGTTTCTCCTAAATTCCGACCATTAAACCTCATTA-3'. Hot probes were ordered pre-biotinylated on their 5' ends. Whole cell lysate and probe were mixed in EMSA binding buffer (10 mM HEPES pH 7.9, 1 mM EDTA, 60 mM KCl, 7% glycerol) with poly-dI:dC and incubated at room temperature for 45 minutes before electrophoretic resolution on a 6% non-denaturing polyacrylamide gel. Each EMSA reaction contained 14.5 μ L binding buffer (containing poly-dI:dC), 1.5 μ L probe, variable amounts of lysate and dH₂O to bring the total reaction volume up to 20 μ L. DNA/protein complexes were then transferred to a Biotodyne nylon membrane, UV crosslinked for 30 seconds and blocked overnight. Detection of complexes was performed with Chemiluminescent Nucleic Acid Detection Module Kit (Thermo Scientific). Where indicated, lysates were immunoprecipitated with either rabbit α -PPAR γ (Cell Signaling, Clone: 81B1) or Ig control antibodies overnight at 4°C as described above.

2.8 Mice

6-8 week old PINK1 knockout mice were generated on a 129/Sv background⁶⁹ before crossing with C57BL/6 for at least 10 generations. All mouse work was approved by the Institutional Animal Care and Use Committee at the University of Kentucky.

2.9 *in vitro* T cell activation

1 μ g of α -CD3 antibody (Clone: 45-2C11) with or without 500 ng of α -CD28 (clone: 37.51, eBioscience) antibody was added to a single cell suspension of 10⁶ LN

CD4⁺ T cells and incubated for 15 minutes on ice. Bound antibody was then cross-linked with 2 µg of α-IgG for 3 minutes at 37°C and quenched with cold RPMI.

2.10 Extracellular flux analysis

Extracellular flux analysis was performed on an XF-96 bioanalyzer (Seahorse Bioscience) according to manufacturer's protocol. First, XF assay medium was pH balanced and supplemented with both 25 mM glucose and 1 mM sodium pyruvate. 4×10^5 T cells per well were resuspended in XF assay medium and attached to a 96-well plate pre-coated with Cell-Tak (BD Biosciences) for 20 minutes at a concentration of 22.4 µg/mL, followed by gentle centrifugation. During each run, the bioanalyzer injected solutions from each of four separate ports (A-D) into the each well. Because of the additional volume from each injection, oligomycin in port A was formulated at 8X final concentration, (carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone) FCCP in port B at 9X, etomoxir in port C at 10X and rotenone / antimycin A in port D at 11X. Oligomycin was used at a final concentration of 1 µM, FCCP was used at 1.5 µM, etomoxir at 200 µM and rotenone / antimycin A were injected at the same time, each at 1 µM. Samples were either run in triplicate or quadruplicate, and the changes in oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured and compared to background correction wells containing no cells, but still receiving each liquid injection. The bioanalyzer was programmed to make 3 measurements before port A's injection (baseline), 3 measurements after injection of oligomycin, FCCP and rotenone / antimycin A and 5 measurements after etomoxir. Derived perimeters were calculated as follows, with letters corresponding to Figure 4.5A/B: Basal resp. = A-E,

ATP Prod. = A-B, max resp. = C-E, spare respiratory capacity (SRC) = C-A, fatty acid (FA) resp. = C-D, Non-FA resp. = D-E.

2.11 Statistical analysis

Statistical comparisons were made using unpaired, 2-tailed Student's t-test. A p value of < 0.05 was used as the cutoff for statistical significance.

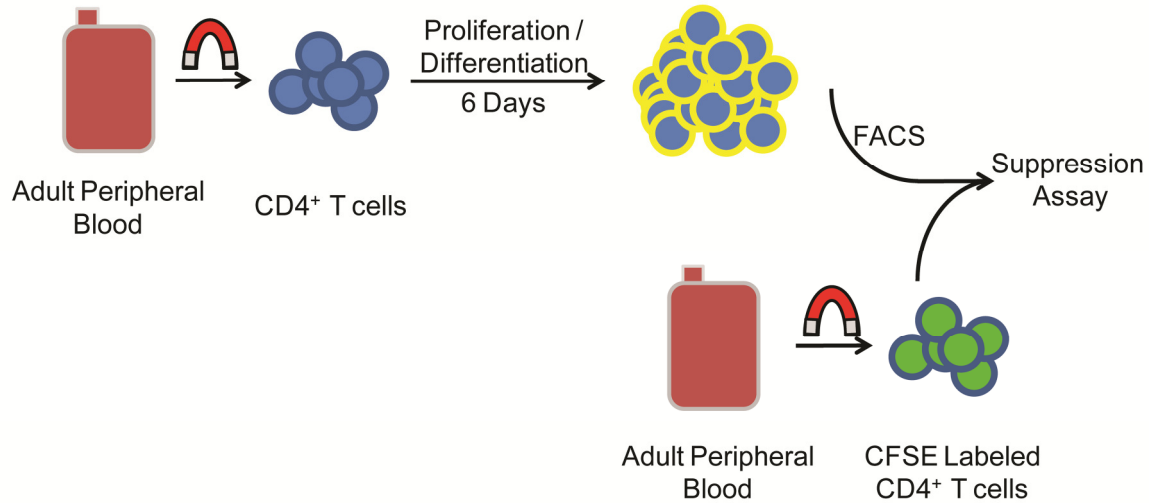


Figure 2.1 Generation of iTregs from human peripheral blood donors. Following isolation of PBMCs from granulocyte samples via gradient centrifugation, magnetic negative separation isolated $CD4^+$ T cells, which were cultured on α -CD3 coated plates in medium containing TGF- β and IL-2. On day 6 of culture, fluorescence activated cell sorting (FACS) separated iTregs ($CD25^{hi} CD45RA^-$) from effector ($CD25^{mid} CD45RA^-$), memory ($CD25^- CD45RA^-$) and naïve ($CD25^- CD45RA^+$) T cells. To test suppressor function, each subset was co-cultured with CFSE labeled, autologous T cells and α -CD3/ α -CD28 beads, before flow cytometric analysis of CFSE dilution.

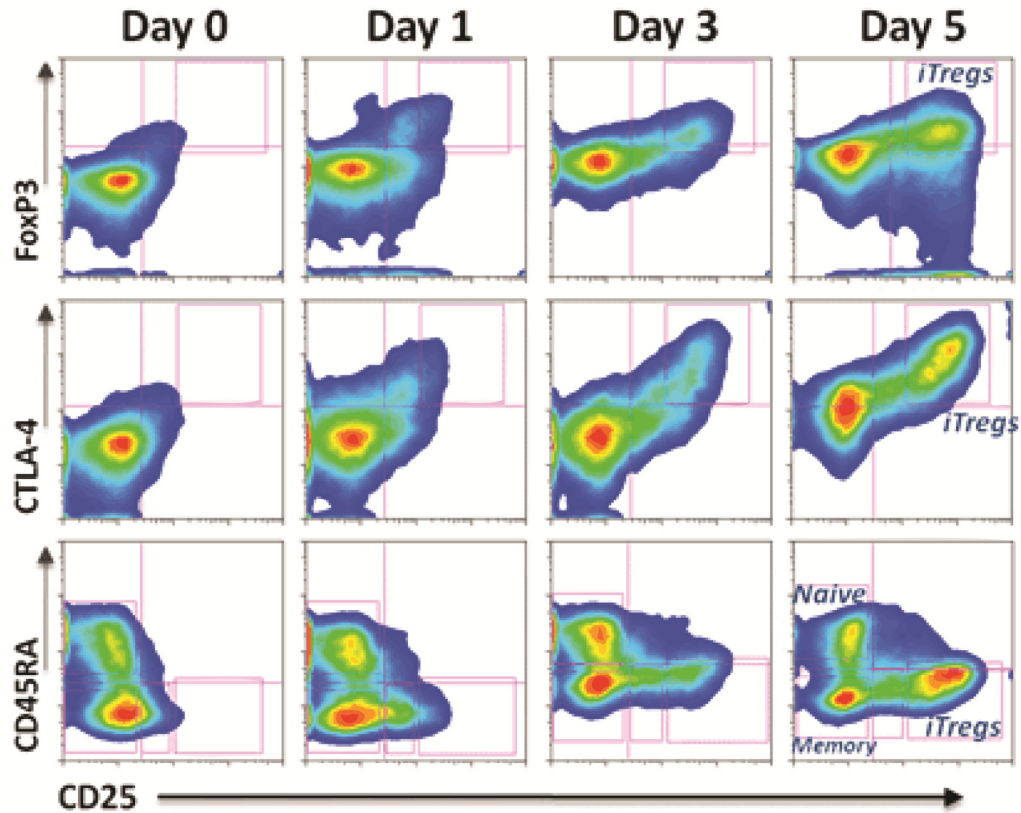


Figure 2.2 Representative plot of iTreg differentiation from CD25⁻ human donor cells. Flow cytometric pseudocolor dot plots demonstrate the kinetics of iTreg development as generated in Figure 2.1. iTreg yield fluctuated from 5%-40% depending on donor variability and inclusion of ligands in the culture medium.

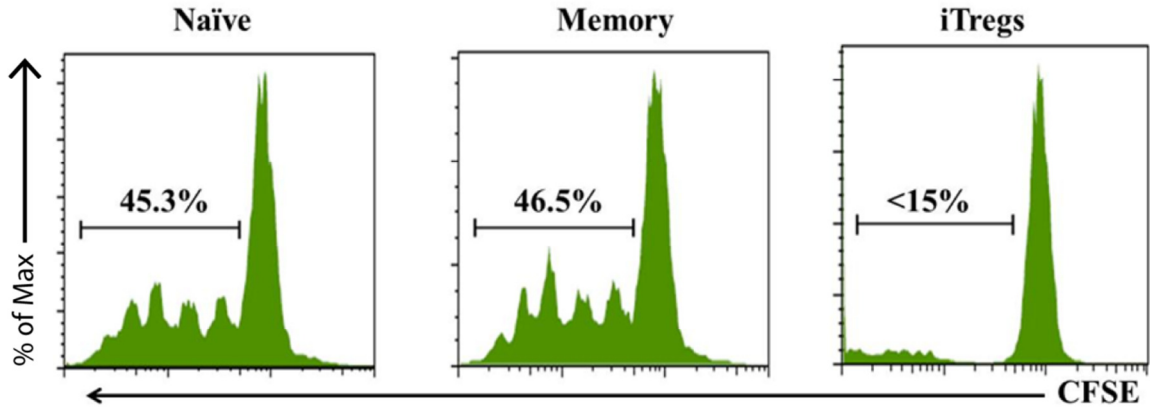


Figure 2.3 The iTreg subset (CD25^{hi} CD45RA⁻) has demonstrable suppressor activity. Cultured CD4⁺ T cells were sorted into naïve (CD25⁻ CD45RA⁺), memory (CD25⁻ CD45RA⁻) or iTreg (CD25^{hi} CD45RA⁻) subsets and incubated with CFSE labeled, autologous CD4⁺ T cells for 5 days. Each CFSE peak represents one cell division. Histograms above are representative of 3 human experiments performed at a ratio of 3 CFSE labeled T cells to 1 iTreg, and represent the general trend expected to occur in healthy humans and wild type mice when probing the suppressor activity of functional iTregs.

Chapter 3: Human iTreg development is enhanced by the T cell autonomous all-trans retinoic acid synthesis pathway mediated by PPAR γ

3.1 Introduction

Aside from the TCR, co-stimulatory and cytokine signals received during priming, a T cell must integrate signals from other molecules that may be in the vicinity to further refine the T cell response. Nuclear receptors, including peroxisome proliferator-activated receptor- γ (PPAR γ) and retinoic acid receptor- α (RAR α), are a family of ligand inducible transcription factors that can execute that need by responding to signals from fatty acids, eicosinoids, prostaglandins and dietary ligands (PPAR γ) or retinoids (RAR α).

The effects of PPAR γ on downstream transcription are enacted by several distinct mechanisms⁷⁰. Generally, it traffics to the nucleus where it constitutively binds a PPAR γ response element (PPRE) half site via its zinc finger DNA binding domain and heterodimerizes with the retinoid X receptor (RXR). In this basal, non-ligand bound state, PPAR γ 's transactivation domain recruits a complex of transcriptional co-repressors to downregulate the expression of responsive genes. Agonist binding induces a conformational change that stabilizes the molecule's AF2 domain, and encourages a switch of the co-repressors for co-activators, many of which are then sequestered from the pro-inflammatory NF- κ B. Alternatively, PPAR γ can negatively regulate other transcription factors through transrepression by mechanisms that are still becoming clear. In macrophages, LPS induces transcriptional activation of *iNOS* by a similar exchange of co-repressors for co-activators. In the presence of ligand however, PPAR γ is SUMOylated and trafficked to the *iNOS* promoter where it prevents dissociation of co-

repressors⁷¹. In the gut, the commensal species *Bacteroides thetaiotamicron* attenuates inflammation by directing PPAR γ to shepherd the RelA subunit of NF- κ B out of the nucleus and into the cytoplasm⁷². The multiple mechanisms of action, diversity of targets and non-specific effects of its ligands have made PPAR γ a relatively difficult molecule to study.

Though several endogenous ligands have been proposed for PPAR γ including eicosanoid 15-deoxy- $\Delta^{12,14}$ prostaglandin J₂ (15d-PGJ₂), poly-unsaturated fatty acids and oxidized low-density lipoproteins⁷³, these molecules were not found in high enough concentrations *in vivo* to be considered physiologically relevant modulators. Still, PPAR γ has been an active target of pharmaceutical intervention with synthetic ligands. Members of the thiazolidinedione (TZD) class of drugs including pioglitazone, ciglitazone, rosiglitazone (RSG) and troglitazone have been used to treat type 2 diabetes⁷⁴ due to PPAR γ 's ability to decrease insulin resistance, but are associated with congestive heart failure⁷⁵ among other side effects, which have caused their removal from the market.

Transcriptional roles for PPAR γ have been noted in the control of inflammation, metabolism, and adipogenesis⁷⁶. PPAR γ stimulation by TZDs induces the differentiation of pre-adipose cells⁷⁷, positioning PPAR γ as the master regulator of adipogenesis. In dendritic cells and macrophages, PPAR γ activation induces an anti-inflammatory phenotype by influencing a range of characteristics^{71,78,79}. For one such change in human dendritic cells, treatment with RSG increased the expression of several molecules involved in the metabolism of vitamin A (retinol) into its transcriptionally active form, all-trans retinoic acid (ATRA). Vitamin A has a long established role in the proper function and development of the immune response⁸⁰. As part of the gut immune system,

CD103⁺ dendritic cells³⁵, intestinal epithelial cells⁸¹, mesenteric lymph node stromal cells⁸² and CD14⁺ macrophages⁸³ possess the machinery to metabolize and secrete ATRA, in addition to other extra-mucosal tissues such as testicular Sertoli cells⁸⁴. The presence of ATRA during naïve T cell priming synergizes with TGF- β to increase the development, stability and relative suppressor strength of iTregs^{35,85-87}. ATRA also actively represses the expression of ROR γ T, the master transcription factor of the Th17 lineage⁸⁸ and induces gut homing molecule expression⁸⁶. When provided ATRA by CD103⁺ dendritic cells or from other sources, lymphocytes upregulate expression of the chemokine receptor CCR9, which causes trafficking to the CCL25 producing areas of the small intestinal crypt regions, and the integrin $\alpha_4\beta_7$, which binds to MAdCAM-1 expressed on blood vessel endothelial cells of the intestinal, respiratory and urogenital tracts⁸⁹.

Retinol is converted to ATRA via a 2 step process: retinol is reversibly oxidized into retinal by a member of the short-chain dehydrogenase/reductase (SDR) or alcohol dehydrogenase (ADH) families as the rate limiting step, and retinal irreversibly becomes ATRA via a secondary oxidation by a retinaldehyde dehydrogenase (RALDH). ATRA binds to RARs, which form obligate heterodimers with RXRs at retinoic acid response elements (RAREs) to modulate transcription. RDHs are largely thought to have redundant function because of their wide range of expression patterns and overlapping function⁹⁰, so much of the focus on pathway regulation has been on the more tissue-specific expression of RALDHs. However, recent work has shown that retinol dehydrogenase 10 (RDH10), an SDR, is non-redundantly required for embryonic development and post-natal viability and spermatogenesis⁸⁴, despite the expression of

other ADHs⁹¹. The sub-cellular localization of RDH10 to lipid bilayers gives it exclusive access to a pool of retinol contained within retinol binding protein-free membranous vesicles⁹².

Since PPAR γ activation trends towards an anti-inflammatory response and its expression is necessary for murine iTreg development⁹³, we reasoned that human iTregs might be similarly regulated, giving them the ability to respond to similar external signals. In this chapter, we confirm that T cell expressed PPAR γ is important for human iTreg development, and that its ligand-induced activation endows iTregs the ability to autonomously metabolize retinol into endogenous ATRA via the direct binding of PPAR γ to the *RDH10* locus.

3.2 Results

3.2.1 PPAR γ is necessary for optimal human Treg development

To determine which transcription factors could play a role in the development, stability and/or function of iTregs, we performed a microarray analysis comparing gene expression levels from iTregs, memory, naïve and effector T cells within individual human donors. Upon examining the list of transcription factors with a greater than two fold enrichment in iTregs versus CD25⁻ T cells (Figure 3.1A), we noticed an upregulation of *Pparg* (Figure 3.1B), which we confirmed at the protein level (Figure 3.1C). Since PPAR γ has genomic and non-genomic function, we also demonstrated that the PPAR γ expressed in iTregs and naïve T cells has the ability to bind a consensus PPRE (Figure 3.1D). As a conditional PPAR γ knockout in murine CD4⁺ T cells was correlated with fewer Tregs in mice⁹⁴, we examined the role of this nuclear receptor in human iTreg generation. Though addition of the PPAR γ agonist RSG to culture medium had no

enhancing effect on iTreg differentiation, pharmacologic inhibition with GW9662 subdued iTreg development (Figure 3.1E/F).

3.2.2 Retinol enhances iTreg generation in human CD4⁺ CD25⁻ T cells downstream of PPAR γ

A further investigation of our microarray analysis revealed an enrichment in the levels of *RDH10* within the iTreg subset and a corresponding decrease in *DHRS3* (the SDR1 gene), an antagonistic enzyme which preferentially catalyzes the reduction of retinal back to retinol⁹⁵. In addition, the cytochrome P450 family member *CYP3A5*, which degrades ATRA into 4-hydroxy-retinoic acid⁹⁶ was reduced, and expression of *RARA* was increased (Figure 3.2A). The summation of these genetic changes creates an overall intracellular environment favoring the synthesis, stability and signaling ability of ATRA. As multiple immune and structural cell types in the gut produce ATRA, we asked whether this machinery would endow iTregs with the ability to perform this function intrinsically. The inclusion of either retinol or retinal into the cell culture medium increased iTreg development with potency equal to that of ATRA at equimolar concentrations (Figure 3.2B). Similar studies performed with ATRA in mouse and human systems suggest that ATRA responsiveness is restricted to naïve T cells^{85,97}, which holds true in our iTreg differentiation platform, where we used magnetic negative separation to isolate T cells enriched for either memory or naïve T cells (Figure 3.2C). The stimulatory effect of retinol on naïve T cells could be abrogated by simultaneous addition of the RALDH antagonist citral or RAR α antagonist RO 41-5253, demonstrating the importance of RAR α signaling over RAR-independent effects (Figure 3.2D/F). The iTreg stimulating properties of retinol agonism were not affected by GW9662, situating the

RAR α pathway downstream of PPAR γ (Figure 3.2E/F). These results suggest a functional T cell intrinsic ATRA synthesis pathway in naïve T cells downstream of PPAR γ and mediated by changes in gene expression of the RAR α pathway.

Because RSG treatment induced *RDH10* expression and ATRA secretion in human dendritic cells and ATRA rescues iTreg differentiation after PPAR γ blockade, we cultured sorted, CD4⁺ T cells in iTreg medium containing either RSG and 9-cis RA to stimulate PPAR γ , GW9662 and 9-cis RA to inhibit PPAR γ or vehicle control. After 24 hours of stimulation, PPAR γ stimulated T cells exhibited a 6-fold increase in *RDH10* transcript levels compared to vehicle control, indicating the conservation of a PPAR γ to *RDH10* pathway similar to that in other cell types (Figure 3.2G).

3.2.3 PPAR γ increases transcription of *RDH10* via direct binding

The pleiotropic nature of PPAR γ leaves doubts as to whether its effect on iTreg development and *RDH10* induction is direct or indirect. Upon examination of the *RDH10* gene, we found an evolutionarily conserved PPRE half site slightly downstream of exon 2, which we thought might allow PPAR γ binding (Figure 3.3A). T cell lysates were able to bind to a biotinylated double stranded DNA oligonucleotide probe representing the putative PPRE and its surrounding nucleotides in an EMSA. A wild type cold competitor, but not one in which the PPRE had been altered, was able to compete away binding of the biotinylated form, demonstrating the necessity of the PPRE for binding (Figure 3.3B). Since we used a whole T cell lysate, our experiment said nothing about the identity of the protein bound to the probe. Immunoprecipitation of the lysate with an α -PPAR γ antibody eliminated one of the bands, identifying a PPAR γ -containing complex as directly binding to this site (Figure 3.3C).

3.3 Discussion

In this chapter, we establish the importance of PPAR γ to human iTreg development, and demonstrate that the PPAR γ /RDH10 transcriptional pathway seen in dendritic cells works similarly in T cells, imparting the ability to synthesize ATRA autonomously. As ATRA is only found at very low levels in serum, only un-metabolized forms are available for cellular uptake. In order to signal through the RAR/RXR heterodimer, a cell must first oxidize retinol to ATRA or have it provided by a cell type which can catalyze this conversion. When an iTreg strays from ATRA-producing CD103⁺ dendritic cells and moves to sites other than organized lymphoid organs, its expression of RDH10 endows it the ability to metabolize its own ATRA. Since we have not demonstrated that iTreg-produced ATRA has the ability to leave the cell, this activity may serve as a mechanism whereby iTregs stabilize their own identity. If iTregs have the ability to provide ATRA to other cell types as DCs do, close proximity of cells is most likely required. T cells have the ability to interact with lymphoid cells such as non-ATRA producing APCs, B cells, and other regulatory and non-regulatory T cells in addition to non-lymphoid cells. Following, iTreg-produced ATRA could support intestinal homeostasis by inducing the proliferation, mucosal homing and IgA class switching of Peyer's patch and peripheral B cells⁸⁹ or supporting the maintenance of the intestinal epithelial barrier where PPAR γ expression is high⁹⁸.

In terms of T cell immunity, ATRA producing T cells more than likely play important roles in gut immunity and oral tolerance. iTregs secreting both TGF- β and ATRA engaging in homotypic interactions with resting T cells⁹⁹ could impart infectious tolerance upon the latter. Mucosal iTregs which recognize food peptide could induce

tolerance for the same antigens when found in the periphery, suppressing pro-inflammatory responses and inducing iTreg differentiation of other T cells. In a PPAR γ deficient mouse model of colitis, both vitamin A deficient and vitamin A high diets were more protective compared to a diet at the normal dietary range¹⁰⁰. Anti-colitic Tregs in the former model were CD103⁺ CCR7⁺ and correlated with increased thymic Treg output, while those in the latter expressed CCR9 and $\alpha_4\beta_7$ and trafficked better to the small intestine. Therefore, the tight control of retinoic acid is essential for proper gut immunity. In addition to its effect in a metabolized state, retinol has other ATRA-independent effects on gene expression. In studies where mice are put on retinol-free diets, supplementation with ATRA rescues some, but not all effects including spermatogenesis¹⁰¹, and retinol is required for B cell growth¹⁰². Vitamin A can also tilt the balance of the Th1/Th2 axis by downregulating Th1 responses and supporting Th2 immunity, which has been suggested to be a key component in the prophylactic effect of helminthic therapy for IBD¹⁰³.

Though the role of retinoids in the Treg response has been widely demonstrated, a complementary hypothesis posits that ATRA could be a general proliferation and differentiation factor, based on work detailing its role in promoting pro-inflammatory responses against *Toxoplasma gondii*¹⁰⁴. In addition, low doses of ATRA have shown properties of adjuvanticity: IL-15 primed tolerogenic DCs to produce IL-12 and IL-23 which synergize with ATRA to block iTreg formation and promote the Th1 and Th17 subsets¹⁰⁵. Therefore, it seems as if the context in which ATRA is presented to T cells will determine the direction of ATRA's differentiation-inducing effect, where one would

expect the recognition of pathogen associated molecular patterns to influence the direction of the response to oral antigen.

Despite the importance of RDH10 to pre-natal development and post-natal viability, viable *RDH10*^{-/-} mice have been generated by rescuing the phenotype with maternal dietary supplementation of all-trans retinal¹⁰⁶. These mice exhibit truncated forelimbs and a characteristic “spinning” phenotype, consistent with the role of RDH10 in hindbrain development¹⁰⁷. In the absence of any immunological challenge, these mice have not been reported to have any immunological defects including colitis. However, a preliminary experiment of ours did demonstrate a consistent accumulation of CD19⁺ B cells in peripheral lymph nodes isolated from heterozygous mice expressing only one functional *RDH10* allele (data not shown), potentially due to reduced mucosal trafficking. We believe that further delving into the immunological phenotype of full RDH10 knockout mice in situations of active inflammation will reveal a unique role for RDH10 in immunosuppression. Though the ability to synthesize retinoic acid from retinol is shown here to be a novel pathway for T cells, quite a bit of redundancy exists within hematopoietic and non-hematopoietic cell types, especially in the gut. More often than not, duplications in systems provide a failsafe mechanism to guard against dysfunction, which we believe is the case here, as loss of intestinal homeostasis invariably causes inflammation, discomfort and reduced nutrient absorption needed to sustain life. However, autonomously produced endogenous ATRA likely has a few functions unique to T cells. To tease apart the *in vivo* function of this pathway in T cells, we have recently generated a mouse which lacks RDH10 expression in all CD4⁺ and CD8⁺ T cells (CD4-Cre, *RDH10*^{F1/F1}) mice. Experiments involving mouse models of infection, oral tolerance

and colitis are ongoing. Although non-functional PPAR γ is only associated with metabolic disorders and diabetes in humans, CD14⁺ intestinal macrophages in healthy controls express significantly higher levels of RDH10 than those isolated from Crohn's disease patients⁸³. This result highlights RDH10's role in intestinal homeostasis, as its absence in certain cell types is associated with disease.

The identification of which specific PPAR γ -induced effects are anti-colitic and which cell types are its effectors should increase therapeutic efficacy and decrease side effects. Here, we identify T cells as one such cell type, and their differentiation into iTregs as the PPAR γ -regulated process which could be targeted by iTreg therapy. Several reports have proposed PPAR γ as a therapeutic target in IBD^{108–110}, as mice harboring PPAR γ -deficient enterocytes and hematopoietic cells have exasperated colitis in the TNBS and T cell transfer models⁹³. In human IBD, a susceptibility locus has been preliminarily detected on chromosome 3p26, close to the PPAR γ gene located at 3p25–3p26¹¹¹, and a PPAR γ polymorphism has been found to be underrepresented in a population with Crohn's disease¹¹². Additionally, an rRNA analysis of the gut microbiome identified an underrepresentation of bacteria that produce the fatty acid PPAR γ ligand butyrate in IBD microbiomes¹¹³. These and other factors result in a marked decrease in PPAR γ protein and mRNA levels from colonic biopsies in both Crohn's disease and ulcerative colitis patients that do not extend to levels in peripheral blood¹¹⁴. Though PPAR γ ligands reduce inflammatory cytokine secretion and ameliorate DSS colitis¹¹⁰ in mouse models, the expression pattern and pleiotropy of PPAR γ reduces the ability to target certain cells, and concerns over an increased risk of morbidity and mortality have seen TZDs off the market.

To activate a single, relevant arm of the PPAR γ signaling pathway to reduce off target effects, we envision retinoid therapy as a therapeutic modality to treat autoimmunity, and particularly IBD. Mice on vitamin A high diets (25,000 IU/kg, 10X of normal diet) had more CCR9⁺ gut Tregs in intestinal sites (jejunum, ileum, colon) and a less severe phenotype of spontaneous colitis¹⁰⁰. Oral ATRA has been used successfully to combat acute promyelocytic leukemia (APML), a blood cancer caused by a translocation of the *RARA* gene. However, 26% of those prescribed ATRA develop retinoic acid syndrome, a multi-system morbidity characterized by fever, weight gain, pleural and pericardial effusions, acute renal failure and death in some cases¹¹⁵. Oral retinol therapy could hold greater therapeutic benefit based on its inclusion in a normal diet and the reduction of off target effects mostly limited by the cell-type specific expression of both an RDH and a RALDH. Efficacy could also be boosted by transducing T cells *ex vivo* with an RDH10 lentiviral vector, especially if RDH10 expression is deficient, as it is in CD14⁺ lamina propria macrophages isolated from CD patients⁸³, or with a suicide vector that induces apoptosis if a transferred cell loses FoxP3 expression².

Stimulation of PPAR γ by food antigens¹¹⁶, fatty acid-derived eicosanoids or polyunsaturated fatty acids might serve to signal to the responding cell of fat soluble vitamins in the vicinity, and to prepare the machinery to metabolize the vitamin into a transcriptionally active form. Dietary retinol is ingested from plant sources in a pro-vitamin carotenoid form such as β -carotene, or via animal sources as a retinyl ester. Hydrolytic enzymes either secreted by the pancreas or located in the small intestinal microvilli initially free retinol from its lipid carrier, allowing enterocytic uptake. Once across the epithelial cell barrier, retinol is re-esterified with long chain, unsaturated fats,

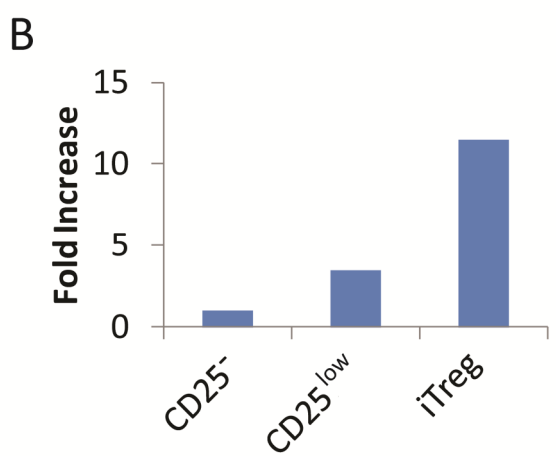
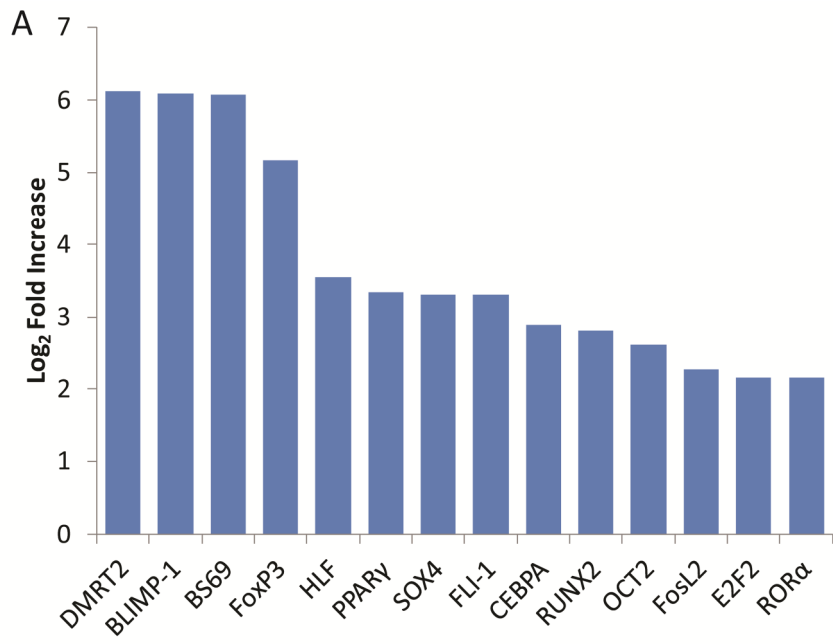
like the PPAR γ -activating linoleic acid^{117,118}, and assembled into chylomicrons. Indeed, increased dietary fat ingestion provides the raw materials for chylomicron formation, and increases retinol uptake¹¹⁷. Unpacking of chylomicrons is a concerted effort: glycosylphosphatidylinositol-anchored high-density lipoprotein-binding protein (GPIHBP1) binds the chylomicron, so triglycerides can then be hydrolyzed into fatty acids by lipoprotein lipase (LPL). Notably, GPIHBP1¹¹⁹ and LPL¹²⁰ are both PPAR γ responsive proteins. Radiotracing experiments show accumulation of dietary retinol in the lymph nodes, and since chylomicrons can also stimulate T cell proliferation¹²¹ needed for iTreg development and transport oral antigen from the gut lumen to the MLNs¹²², this provides a putative mechanism for oral tolerance.

The subcellular localization of RDH10 provides a clue as to how its function may be involved in lipid metabolism, PPAR γ stimulation and mitochondrial oxidative phosphorylation (OXPHOS). In transfected COS7 cells, RDH10-GFP can be found mainly associated with the mitochondrial and mitochondrial-associated membrane (MAM) fractions. The MAM is a subsection of the ER in close enough proximity to mitochondria to influence its function and is the site of lipid droplet biogenesis. Upon the budding off of new lipid droplets, RDH10 leaves the mitochondria and MAM, and instead can be found in the membrane of the nascent lipid droplets¹²³. In the case of retinoid sufficiency, retinyl esters are enveloped in the droplets, which can be mobilized into retinol by membrane associated lecithin retinol acetyltransferase (LRAT)¹²⁴ and then oxidized by RDH10 found in the same lipid monolayer when needed.

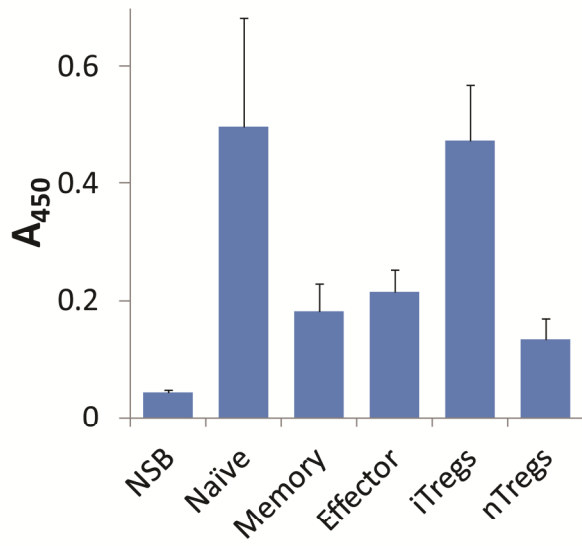
Overall, the work shown in this chapter supports the following model (Figure 3.4). During TCR stimulation in the presence of IL-2 and TGF- β , T cells accumulate

unsaturated fatty acids as part of chylomicrons and/or esterified to molecules of retinol. As ectopic expression of PPAR γ increases triglyceride droplet formation from the ER in fibroblasts¹²⁵ and *Pparg* transduction/stimulation of T cells induces several fatty acid transporter and synthesis genes¹²⁶, we would expect nascent lipids to form within PPAR γ ⁺ iTregs. During this process, PPAR γ upregulates RDH10, which relocates from the ER to the lipid droplet membranes concurrently with LRAT. As in retinal pigmented epithelial cells of the eye, these new lipid droplets will contain retinyl esters, and constitute an intracellular store. Though the role of ATRA in maintaining oral tolerance is well established, contemporary thought does not comment on the fate of tolerized T cells in the periphery. Upon egress from the mucosal immune system, an iTreg encountering its cognate antigen will most likely do so in the context of an ongoing, pro-inflammatory response. In this case, intracellular ATRA and PPAR γ ligands ferried to the site of infection by RDH10⁺ iTregs could impart anti-inflammatory effects upon the initiating innate cells, induce FoxP3 expression in other responding T cell clones which have never entered the mucosal immune system and reinforce a cell's own iTreg phenotype. As each T cell clone in the microenvironment receives slightly different signals depending on the specific APC it interacts with and its location within the body, retinoic acid secretion could be a signal from an individual cell as a vote towards a unified immune response, similar to the "waggle dance" performed by individual honey bees to direct group decision making in the scouting of potential nest sites¹²⁷. For these reasons, it has not escaped our notice that PPAR γ might coordinate feeding, vitamin A ingestion and chylomicron formation as a mechanism to induce oral tolerance, for which $\alpha_4\beta_7$, CCR9 and MLN-to-lamina propria trafficking play a significant role¹²⁸. Future experiments

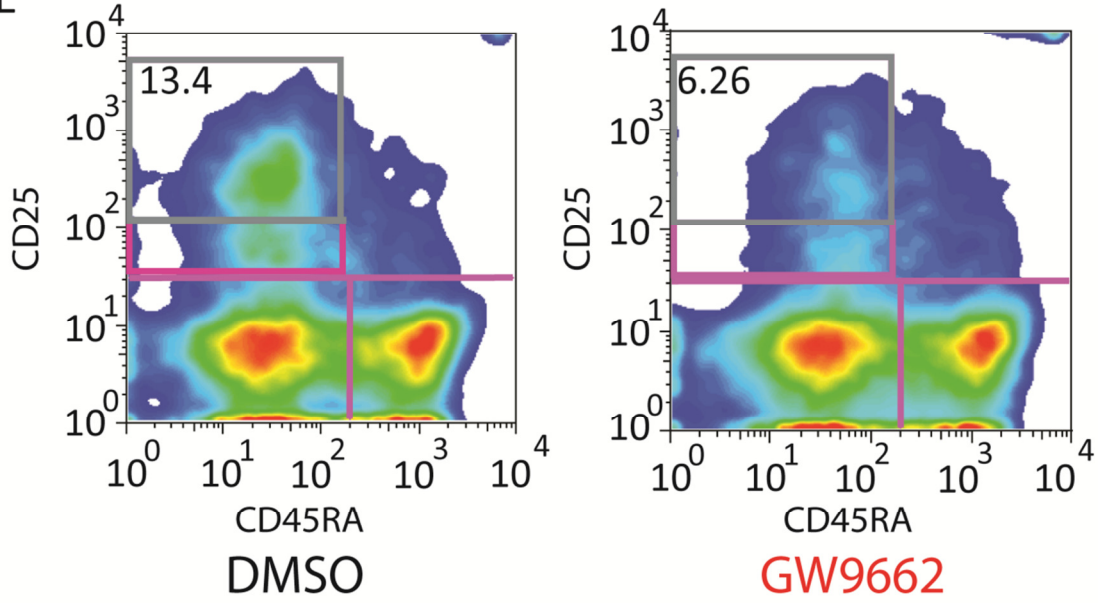
testing the validity of this model will contribute to our knowledge of intestinal iTreg development and aid in the development of novel therapeutics based on this understanding.



D



E



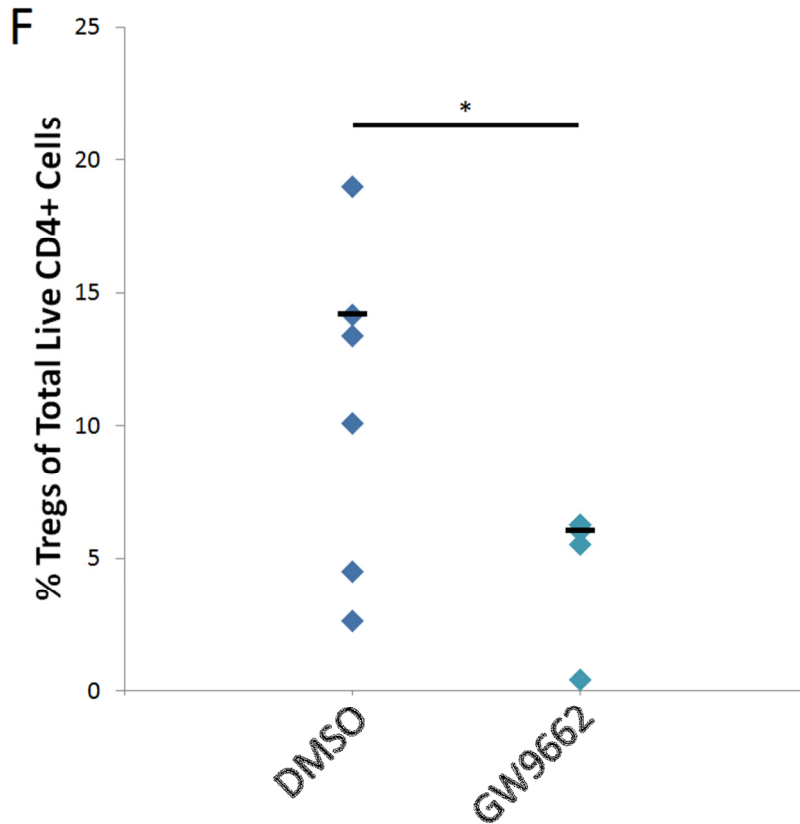
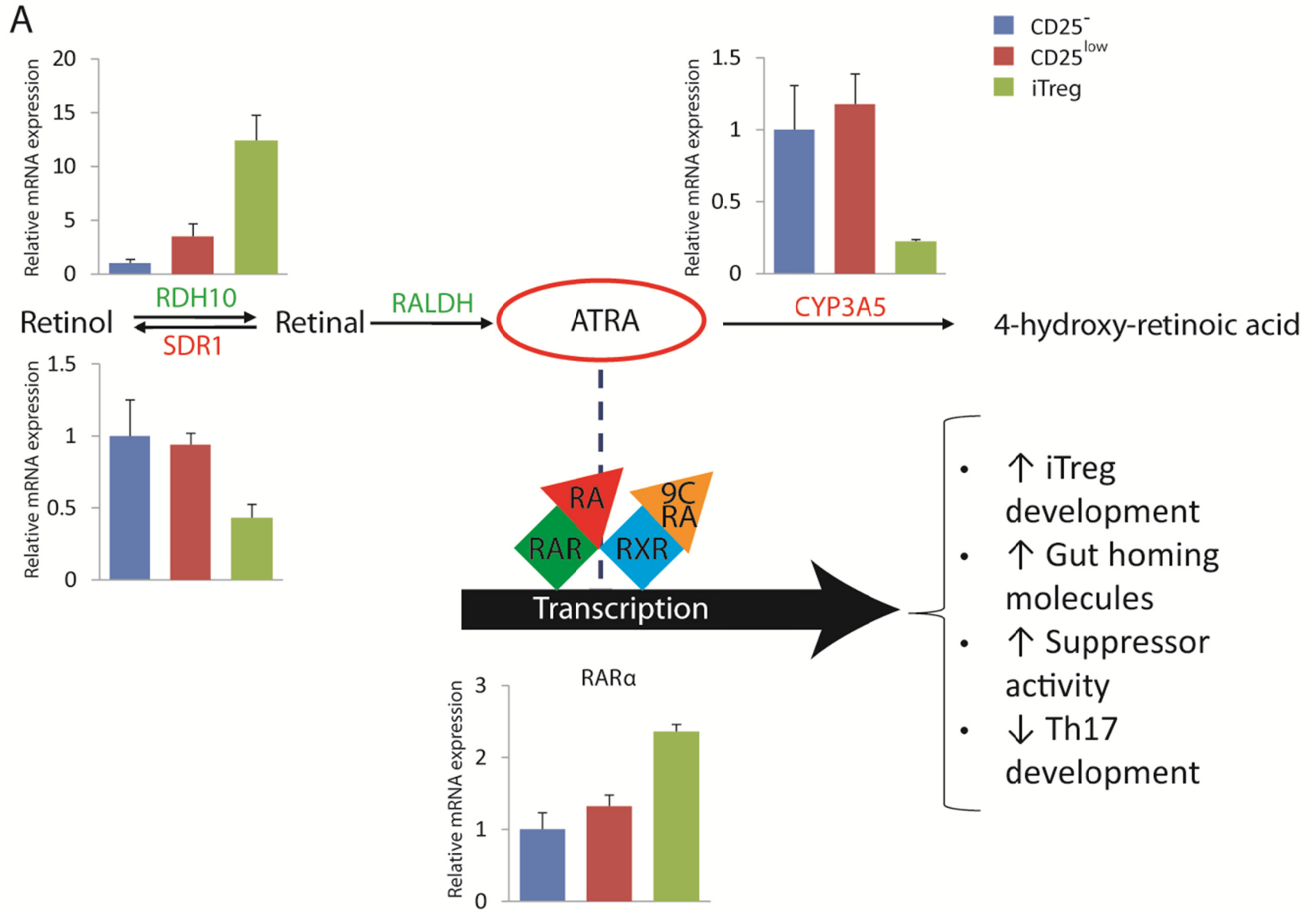
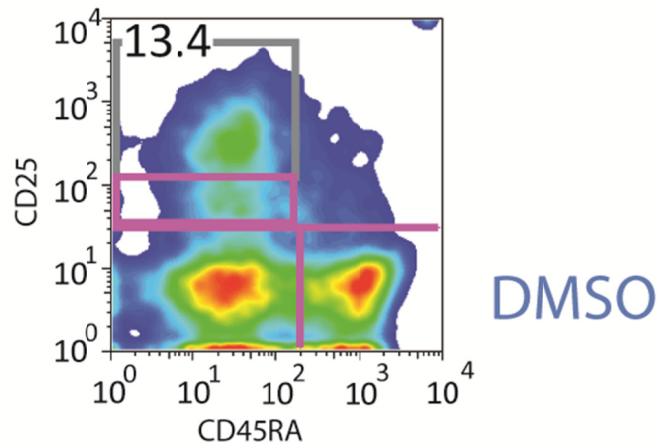
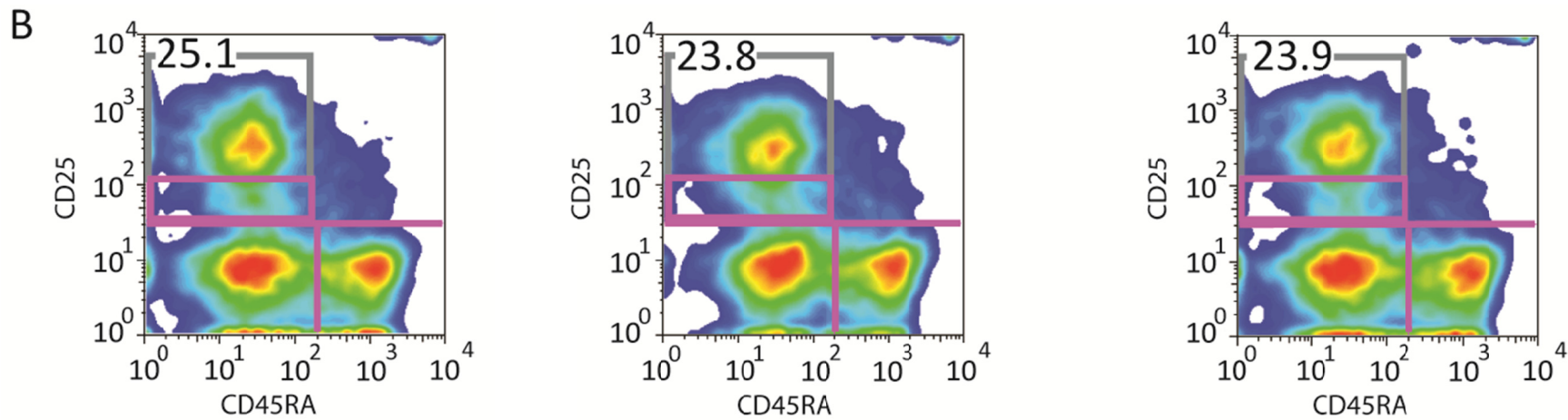
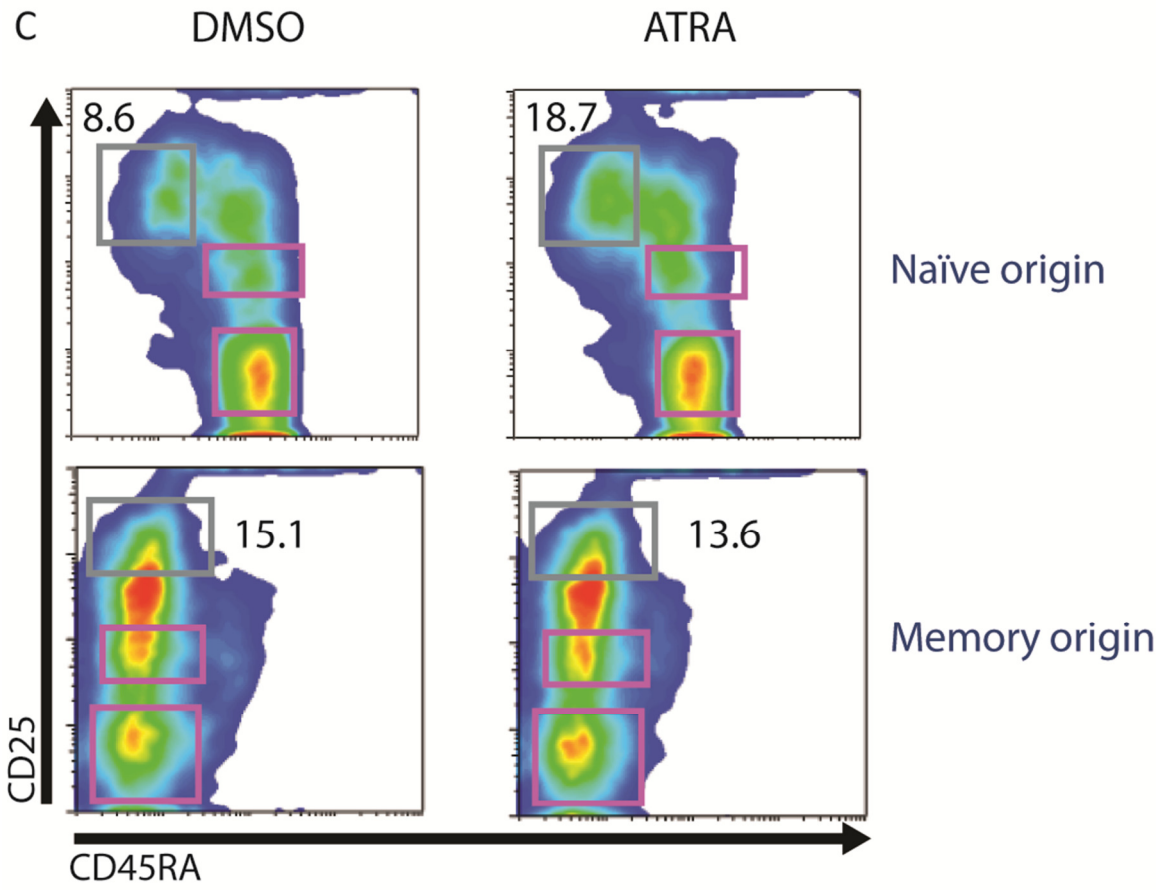
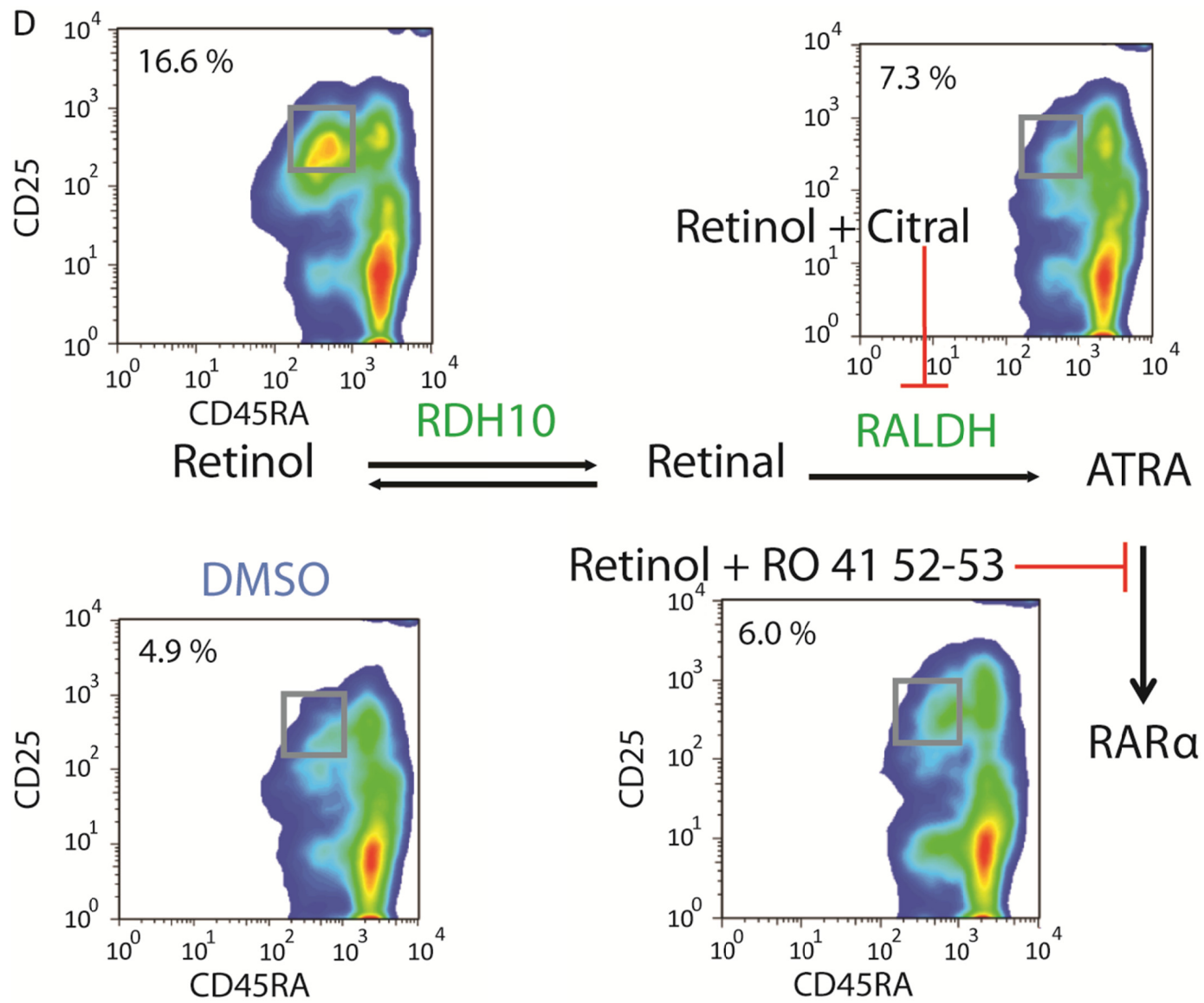


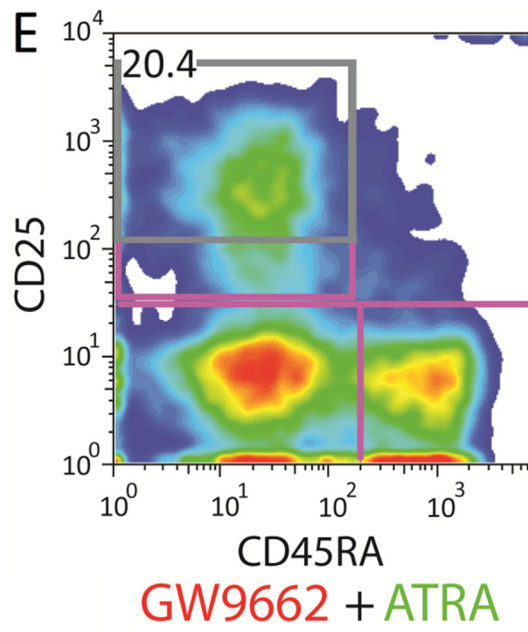
Figure 3.1 PPAR γ is necessary for optimal human iTreg development. (A) Transcription factors with $>2 \log_2$ fold increased expression in sorted iTregs (CD25^{hi} CD45RA⁻) versus CD25⁻ T cells as assessed by microarray. Each of three donor samples was run in triplicate and compared to the other subset within the same individual. Note the increase in *Foxp3* as an internal control. (B) *Pparg* gene expression among CD25⁻, effector (CD25^{low}) and induced regulatory (CD25^{hi} CD45RA⁻) T cells, normalized to CD25⁻ expression levels from the same experiment as 3.1A. (C) Protein expression of PPAR γ from the cell types sorted as in (B) as visualized by Western blot. (D) Nuclear lysates from the indicated cell types were added to individual wells containing fixed PPRE oligonucleotides in an ELISA based assay as measured by 450 nm light absorbance. NSB = negative control. (E) Representative flow cytometry pseudocolor plot of iTreg development over the course of 6 days in culture in the presence of DMSO or 1 μ M GW9662. Number in upper left corner of plot represents the percentage of iTregs (grey box) among all CD4⁺ cells. (F) Percentage of iTreg development from 6 separate, paired human donors from three separate experiments, cultured with either GW9662 or vehicle control (DMSO), * $p < 0.05$, horizontal black bar represents the average value.

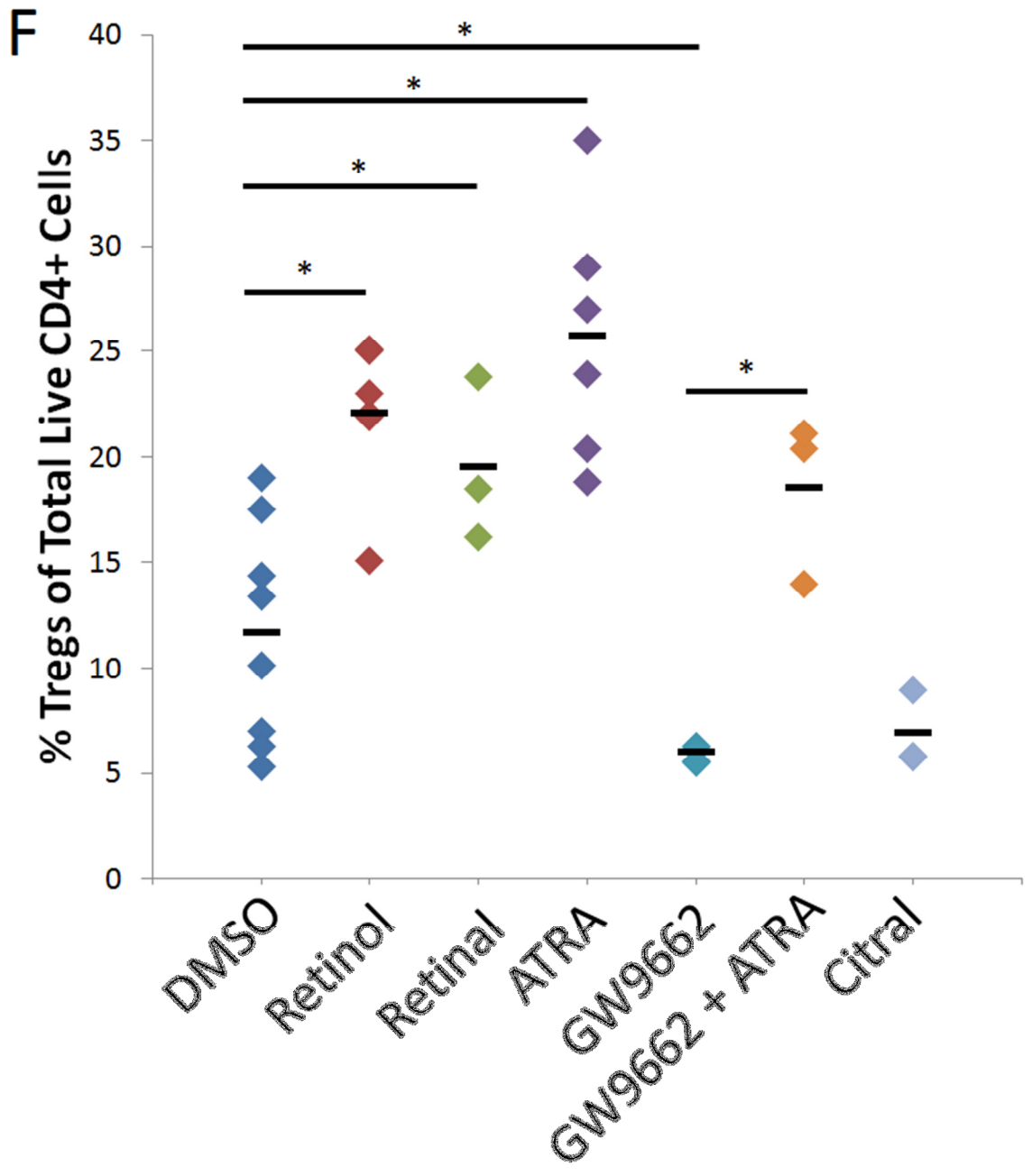












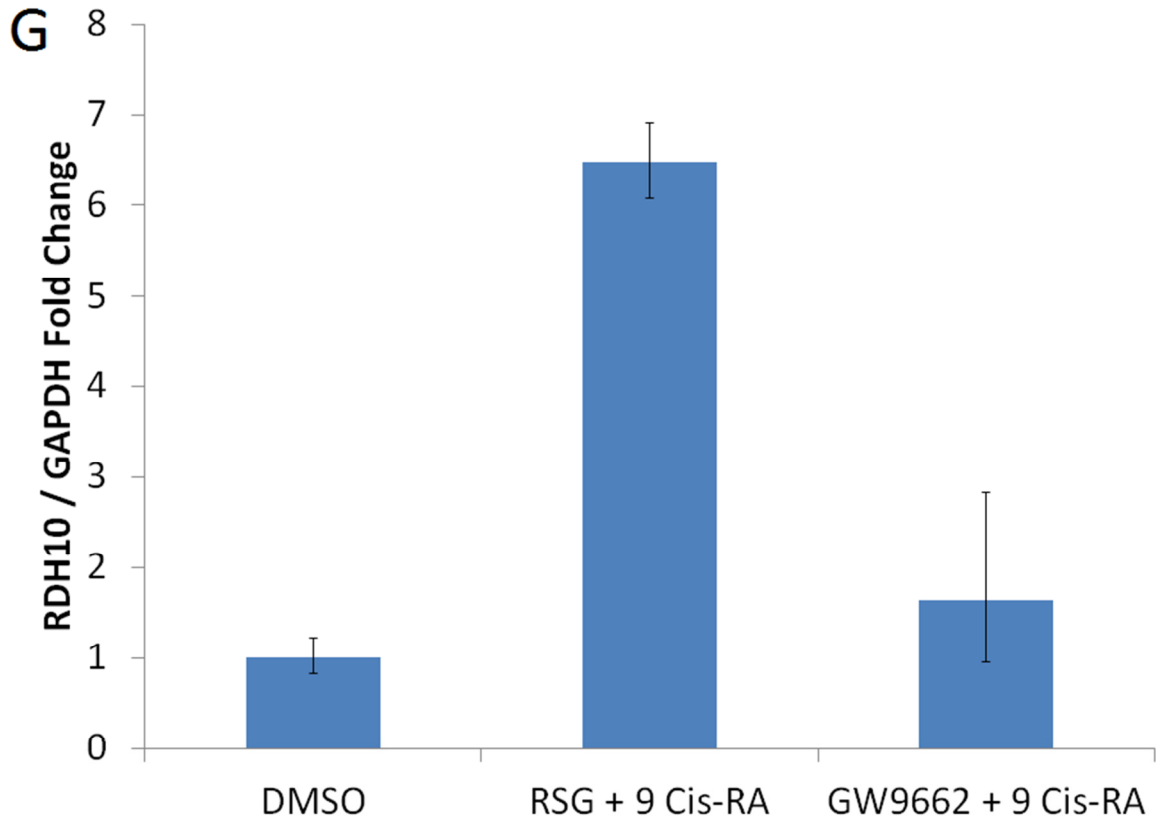
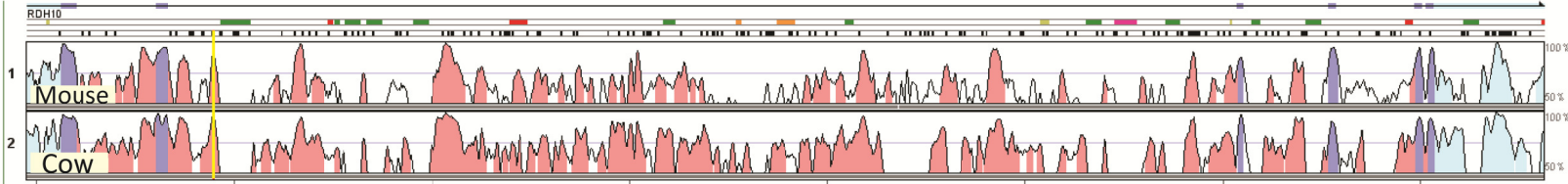


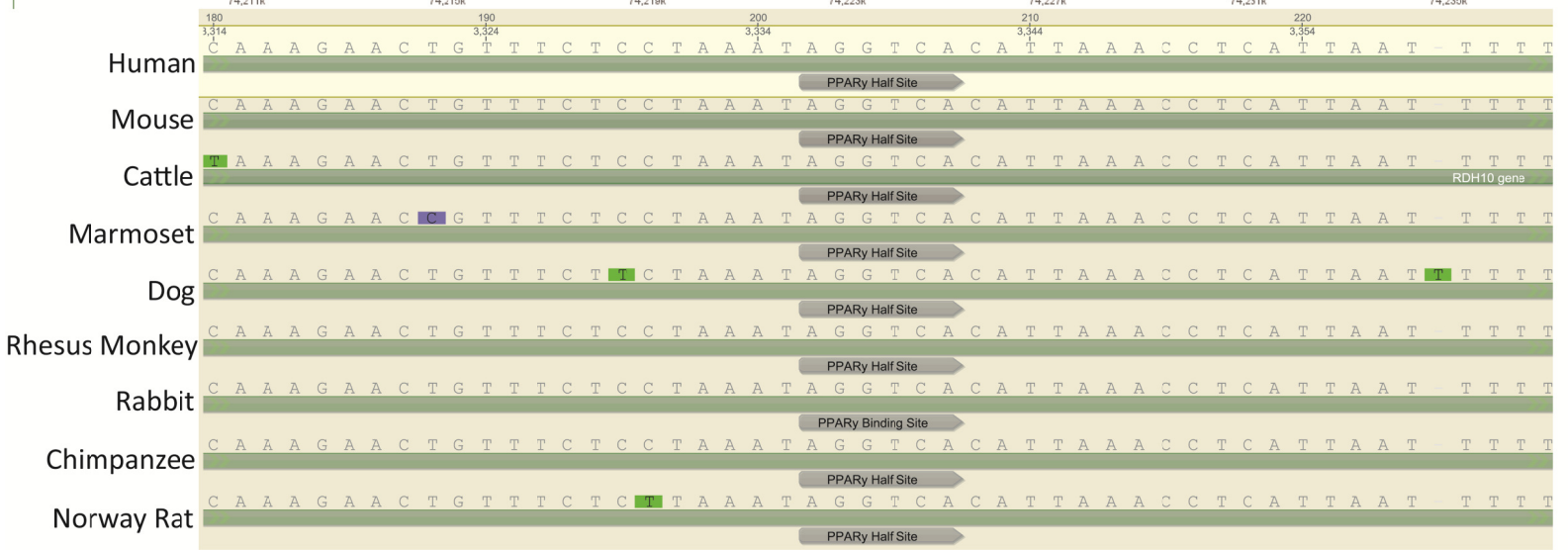
Figure 3.2 PPAR γ controls the endogenous retinoic acid metabolism pathway in human iTregs through RDH10. (A) Microarray analysis of gene expression levels from CD25⁻, CD25^{low} or iTregs (CD25^{hi} CD45RA⁻) for *RDH10*, *DHRS3* (SDR1), *CYP3A5* and *RARA* as performed in Figure 3.1. (B) Human CD4⁺ T cells were cultured in α -CD3 coated wells for 6 days in iTreg medium containing 1 μ M retinol, 1 μ M retinal, 1 μ M ATRA or vehicle control (DMSO). Overlaid number represents the percentage of iTregs (grey box) among all live CD4⁺ T cells. (C) Naïve and memory CD4⁺ T cells isolated from the same donor were cultured in iTreg medium for 6 days with either DMSO or 1 μ M ATRA. (D) Representative flow cytometry pseudocolor plot of naïve CD4⁺ T cells incubated in iTreg medium with retinol (top left), retinol + 5 μ M citral (top right), DMSO (bottom left) or retinol + 1 μ M RO 41-5253 (bottom right) for 6 days. (E) CD4⁺ T cells were incubated in iTreg medium with GW9662 + ATRA for 6 days. (F) Bar graph summarizing all donors from figures 3.1E, 3.2B and 3.2E, n = 3-7 individual human donors from 5 independent experiments, * p < 0.05. (G) *RDH10* expression normalized to *GAPDH* as determined by qRT-PCR after 24 hour incubation with 1 μ M RSG + 1 μ M 9-cis RA or 1 μ M GW9662 in iTreg medium, n = 1 human donor, performed in triplicate, error bars represent the relative quantification minimum and maximum based on the standard error of calculated C_T values.

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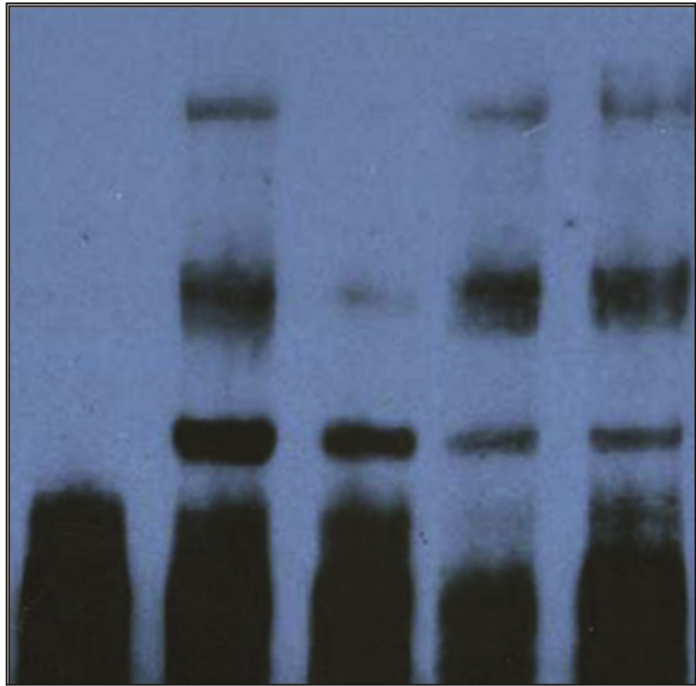
RDH10 Gene



SS



B



Free Probe
1:1 1:10
WT Competitor
1:1 1:10
Mutant Competitor

Biotin Hot WT Probe
CTGTTTCTCCTAAATAGGTCACATTAAACCTCATTA
Cold WT Probe
CTGTTTCTCCTAAATAGGTCACATTAAACCTCATTA
Cold Mutant Probe
CTGTTTCTCCTAAATTCGACCATTAAACCTCATTA

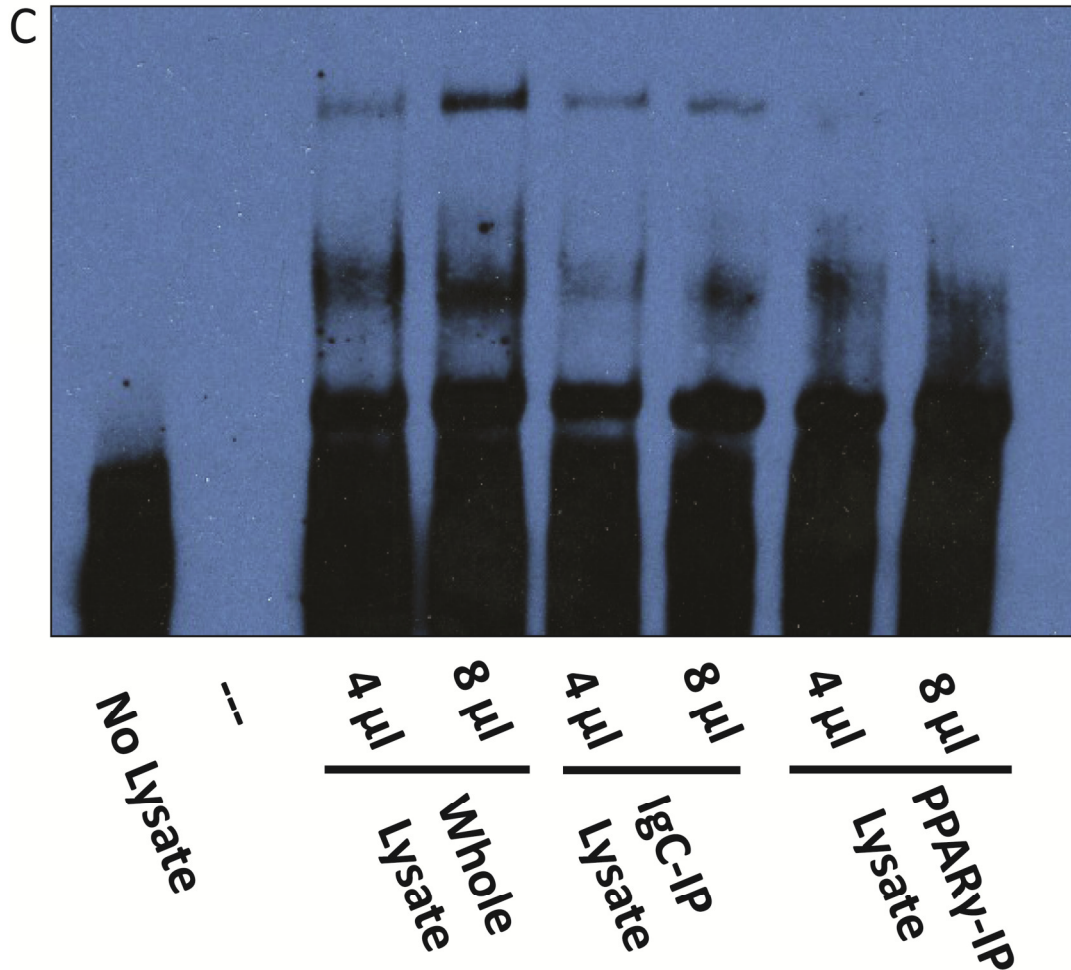


Figure 3.3 PPAR γ directly binds to *RDH10*. (A) Graphical representation of human *RDH10* locus, its exons (black), untranslated regions (white) and its putative PPRE downstream of exon 2 (yellow) (top). Conservation of human, mouse (top) and cow (bottom) *RDH10* loci as visualized by VISTA Browser. Blue sections correspond to exons, putative PPRE designated by a vertical yellow bar on the left side of the figure adjacent to exon 2 (middle). Multiple genome alignment of *RDH10* loci showing nucleic acid level conservation of PPRE site, bases not matching consensus genome sequence are colored. (B) EMSA of human T cell nuclear lysates incubated with probes as shown below the gel, n = 1 human donor. (C) EMSA with identical lysates from (B) pre-immunoprecipitated with α -PPAR γ antibody or IgG control antibody, n = 1 human donor.

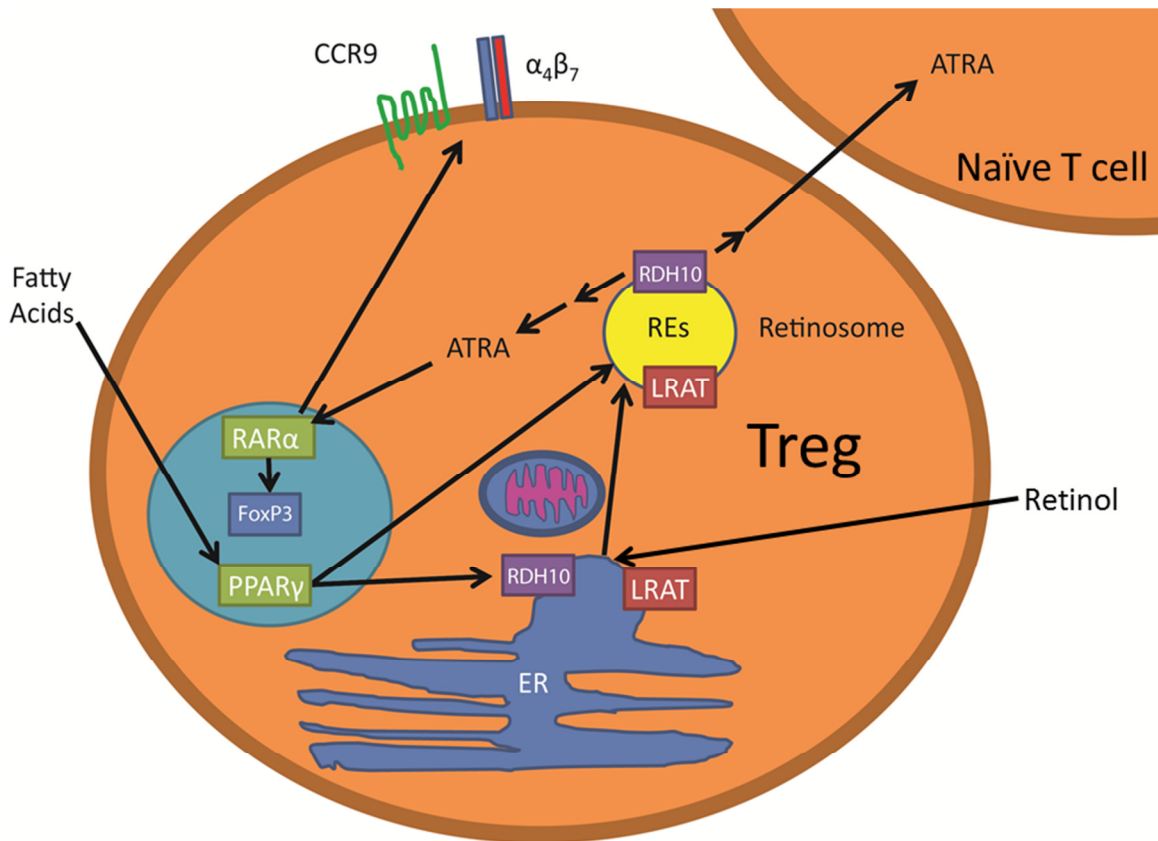


Figure 3.4 Chapter 3 model. Upon TCR stimulation in the presence of IL-2 and TGF- β (not shown), stimulated T cells begin to sense their environment. Fatty acids from the vicinity can stimulate PPAR γ into upregulating expression of RDH10 via direct binding downstream of its exon 2. Membrane bound retinol can then be metabolized by RDH10 and RALDH into ATRA to bind to RAR α and boost FoxP3, CCR9 and $\alpha_4\beta_7$ expression. In addition, PPAR γ stimulation will induce new lipid droplet formation in vesicles containing LRAT and RDH10 already present in the ER, giving LRAT the ability to esterify and store any remaining free retinol into the nascent retinosome to act as an intercellular store in close proximity to RDH10.

Chapter 4: Mitochondrial and cytosolic roles of PINK1 shape iTreg development and function

4.1 Introduction

The activation of a CD4⁺ T cell is an energetically expensive process which encompasses both cellular division and the *de novo* synthesis of proteins and organelles initially, followed by cell fate decision making and acquisition of an appropriate effector phenotype. To support the new energetic needs of an activated, differentiating cell, a rapid re-organization of the cell's metabolic machinery takes place centered on the control of mitochondrial dynamics.

Cells use adenosine triphosphate (ATP) as a form of “molecular currency” to transfer the power stored in its two high-energy phosphate bonds to use as co-factors in enzymatic reactions. In animal cells, ATP is generated from adenosine diphosphate (ADP) and a free phosphate group via a combination of anaerobic glycolysis (lactic acid fermentation) and mitochondrial OXPHOS. Since OXPHOS produces 36 molecules of ATP from 1 molecule of glucose when run to completion compared to only 2 molecules from glycolysis, its efficiency makes it the preferred choice in aerobic conditions when using glucose as an energy source. Without oxygen, cells use fermentation to regenerate nicotinamide adenine dinucleotide (NAD⁺) molecules consumed during glycolysis, resulting in either lactic acid (C₃H₆O₃, eukaryotes) or ethanol (C₂H₅OH, bacteria/yeast). However, Nobel laureate Otto Warburg noted that one of the hallmarks of cancer cells was their seemingly peculiar penchant for generating ATP from the less-efficient aerobic glycolysis despite normoxia, potentially for its ability to generate precursors for nucleic,

amino and fatty acids required for the DNA replication, and protein and cell membrane biosynthesis needed during cellular division^{129,130}.

OXPPOS and lactic acid fermentation both begin via glycolysis – 10 separate chemical reactions break down 1 molecule of glucose into 2 molecules of pyruvate, and generate 2 molecules of ATP via substrate level phosphorylation. In OXPPOS, each of the 2 molecules of pyruvate is then converted into acetyl-CoA by mitochondrial pyruvate dehydrogenase before entering the Krebs cycle (citric acid cycle, tricarboxylic acid cycle), where each molecule of acetyl-CoA produces 3 molecules of NADH. While still in the mitochondrion, OXPPOS and the electron transport chain (ETC) convert the energy stored in reduced pyridine nucleotide into ATP. The process is initiated when NADH is oxidized at complex I, passing 2 electrons to 1 molecule of ubiquinone and pumping 4 H⁺ ions from the mitochondrial matrix to the intermembrane (IM) space. The net change in charge and ion concentration of H⁺ helps establish the proton motive force, an electrochemical gradient between the matrix and the IM space. Complex II, succinate Q-oxidoreductase, is member of both the Krebs cycle and the ETC. Succinate, one of the intermediate products of the Krebs cycle, is oxidized to fumarate and ubiquinone is oxidized to accept 2 electrons. Ubiquinone travels through the lipid bilayer of the mitochondrion to complex III, where 1 electron is passed to cytochrome c, and 4 H⁺ ions traverse the mitochondrial membrane into the IM space. Reduced cytochrome c then advances to complex IV, cytochrome c oxidase. Four molecules of cytochrome c each act as electron donors, becoming oxidized in the process. These four electrons are used to create 2 molecules of H₂O in a reaction where O₂, the terminal oxygen acceptor, is consumed. Four more H⁺ ions are also pumped into the IM space at this juncture to

complete the ETC. Complex V, ATP synthase, uses the potential energy stored by the proton motive force to create ATP from ADP and P_i . For each molecule of ATP generated, 4 H^+ ions are pumped back across the mitochondrial membrane into the matrix (Figure 4.1). As an alternative to mitochondrial OXPHOS described above, pyruvate can be subject to anaerobic fermentation in the absence of oxygen. In eukaryotic cells, NAD^+ is regenerated, and lactic acid and CO_2 are produced as byproducts. Lactic acid production increases the number of H^+ ions secreted by the cell, decreasing the pH of the surrounding environment¹³¹. Importantly, by monitoring the changes in concentration of molecular oxygen from the surrounding medium (oxygen consumption rate, OCR), we can measure the amount of mitochondrial OXPHOS taking place in a given group of cells, and by evaluating changes in pH, we can simultaneously measure glycolysis.

In addition to glucose as an energy source, cells can catabolize fatty acids and amino acids to generate ATP. Fatty acids undergo β -oxidation (fatty acid oxidation, FAO) in mitochondria and peroxisomes to release the energy stored in those molecules. Because of their aliphatic nature, long chain fatty acids (>12 carbons) require activation followed by carrier mediated transport across the outer mitochondrial membrane (OMM) by a carnitine acyl transferase like carnitine palmitoyltransferase-1 (CPT-1) and across the inner mitochondrial membrane (IMM) by a translocase to be oxidized. Their hydrocarbon chains are converted into molecules of acetyl-CoA two carbons at a time, which then feeds into the Krebs cycle and oxidative phosphorylation.

Recent work has noted the occurrence of Warburg metabolism in several leukocytic cell types, with a general trend towards pro-inflammatory or activated leukocytes preferring glycolysis, and anti-inflammatory cell types utilizing OXPHOS.

Neutrophils robustly engage in aerobic glycolysis¹³² to engage in the pentose phosphate pathway, an offshoot of glycolysis needed to rapidly generate the NADPH necessary for NADPH oxidase to produce one of its main antimicrobial effector molecules, H₂O₂. Stimulation of dendritic cells by TLR agonists¹³³ or the B cells by B cell receptor crosslinking¹³⁴, induces a glycolytic shift dependent on PI3K and Akt. Within the dendritic cell realm, aerobic glycolysis is limited to iNOS⁺, pro-inflammatory DCs¹³⁵. Similarly, pro-inflammatory, iNOS⁺ M1 macrophages depend on aerobic glycolysis¹³⁶ and iNOS⁻ regulatory M2 macrophages heavily rely on FAO¹³⁷.

Stimulation of naïve and memory T cells induces a rapid change from primarily using FAO and OXPHOS for ATP generation towards a preference for aerobic glycolysis to support a shift from quiescence to activation in addition to actively upregulating cell surface molecules for glucose and amino acid uptake¹³⁸. The relative reliance on each type of metabolism seems to be both a consequence of differentiation, as well as contributing signal towards the effector lineage decision and function. Pro-inflammatory Th1, Th2 and Th17 cells rely on glycolysis much more than their suppressor iTreg brethren, which engage in a balanced mix of metabolic processes¹³⁹. Abolition of glycolysis by supplementing cell culture medium with 2-deoxyglucose, a competitive inhibitor of glucose, increases iTreg development and reciprocally antagonizes Th1, Th2 and Th17 development¹⁴⁰. Alternatively, blocking lipid metabolism by preventing mitochondrial transfer of fatty acids with etomoxir stunts iTreg development with no apparent effect on pro-inflammatory T cell development, whereas promoting lipid oxidation by fatty acid supplementation to the medium has the opposite effect¹³⁹. In experiments where galactose was provided to cells instead of glucose, Th1 polarized cells

produce less IFN γ , demonstrating the link between the glycolytic shift and effector cell function. This deficiency could be important in creating anti-inflammatory tumor microenvironments, where carcinogenic cells undergoing Warburg metabolism readily deplete glucose. In fact, co-culture of T cells with tumor cells has the same IFN γ -muting effect, which can be rescued with the supplementation of exogenous glucose¹⁴¹.

Therefore, the regulation of mitochondrial metabolism dynamics and their overall health can regulate T cell fate decision and function, especially upon a T cell's migration from a nutritionally replete secondary lymphoid organ to a potentially deplete and hypoxic site of inflammation.

PTEN-induced kinase 1 (PINK1) is a ubiquitously expressed serine/threonine kinase found both at the mitochondrion and in the cytosol. In the absence of PINK1's kinase activity, individuals develop hereditary, early-onset Parkinson's disease (PD)¹⁴². In cells with healthy mitochondria, its N-terminal mitochondrial localization signal targets PINK1 to the IMM¹⁴³, where it is cleaved by the protease Presenilin Associated, Rhomboid-Like (PARL)^{144,145}. Though the binding of protein mitochondrial localization sequences to the translocase of the OMM (TOM) complex is receptor-driven, the import process is critically dependent on forces established by the mitochondrial membrane potential ($\Delta\psi_m$)¹⁴⁶. Upon a decrease in $\Delta\psi_m$, indicative of poor mitochondrial health and reduced proton motive force, PINK1 accumulates on the surface of the individual mitochondrion. PINK1 then recruits parkin, an E3 ubiquitin ligase which polyubiquitinates OMM proteins and targets a mitochondrion for mitophagy, a specialized form of mitochondrial autophagy which specifically culls damaged mitochondria for recycling¹⁴⁷. In addition, PINK1 can exist in the cytoplasm, where it

activates the PI3K/Akt/mTOR pathway^{148,149}. Because pro and anti-inflammatory T cell subsets have different energy requirements, and T cell fate decisions can be altered by manipulating its nutritional milieu, we wondered how the lack of functional PINK1 as in some PD patients would affect T cell activation, differentiation and function. By using a PINK1 knockout mouse model, we reveal that an impairment in T cell activation, OXPHOS and IL-2 signaling in iTreg polarizing conditions results in the generation of FoxP3⁺ iTregs without *in vitro* suppressor function.

4.2 Results

4.2.1 Absence of PINK1 attenuates Akt activation in response to TCR stimulation

Our collaborators in the laboratory of Dr. Hansruedi Büeler demonstrated defective Akt signaling in cells lacking PINK1 downstream of the insulin and IGF-1 pathways⁶⁹, which show similarity to the TCR signaling pathway¹⁵⁰. When we crosslinked the TCRs of *PINK1*^{-/-} peripheral lymph node (PLN) T cells, both Western blot (Figure 4.2A) and flow cytometry (Figure 4.2B/C) showed reduced S473-Akt phosphorylation versus their wild type counterparts. Because β -selection of $\alpha\beta$ lymphocytes requires Akt¹⁵¹, we immunophenotyped murine thymocytes to look for developmental defects, and surprisingly found normal numbers and compositions of precursor T cell pools in knockout mice (Figure 4.3), potentially due to functional compensation by other Akt isoforms¹⁵² or the higher levels of mitochondrial content in thymocytes¹⁵³, rendering the cells resistant to impaired mitophagy.

4.2.2 PINK1 is required for full early activation and proliferation of T cells

Deficient TCR signaling in cells lacking PINK1 foreshadowed effects on ensuing proliferation and activation. Indeed, stimulation of *PINK1*^{-/-} T cells with α -CD3/ α -CD28 resulted in few proliferating cells (Figure 4.4A) and low expression of the early activation markers CD69 and CD25 (Figure 4.4B/C). These changes were not associated with differences in production of IL-2 downstream of CD28 signaling, as synthesis was similar between groups (Figure 4.4D). When we analyzed the integrity of the IL-2 signaling pathway, phosphorylation of STAT5¹⁵⁴, pS473-Akt¹⁴⁹, pT308-Akt¹⁴⁹ and FOXO1¹⁵⁵, but not GSK-3 β was impaired, and GLUT1¹⁵⁶ expression was reduced (Figure 4.4E), indicating that some, but not all of the conventional Akt pathway and mTORC2 are less active. Since the PI3K/Akt/mTOR axis is a known regulator of mitochondrial metabolism¹⁵⁷, we determined $\Delta\psi_m$ by measuring the fluorescence intensity of TMRE, a positively charged, lipophilic dye that accumulates in mitochondria proportionally to its $\Delta\psi_m$ ¹⁵⁸. In most non-lymphoid cells lacking PINK1 or expressing a non-cleavable mutant form, basal levels of $\Delta\psi_m$ are decreased compared with wild type cells^{144,159-161}. Surprisingly, unstimulated T cells from *PINK1*^{-/-} mice had similar levels of TMRE fluorescence across central memory, effector memory and naïve subsets of both CD4⁺ and CD8⁺ T cells. However, when activated with α -CD3 + IL-2, but not α -CD3 + α -CD28, membrane potential was decreased in cells lacking PINK1 (Figure 4.4F).

Though TMRE fluorescence is a fine indicator of mitochondrial health and fidelity, it does not directly measure a cell's metabolic prowess. Based on the principles that mitochondrial OXPHOS consumes oxygen from, and glycolysis deposits hydrogen ions into, the surrounding medium, we used a bioanalyzer to measure extracellular flux in real-time with O₂ and H⁺ responsive fluorophores as follows (Figure 4.5A/B). First, three

initial measurements determined the baseline OCR. The bioanalyzer then injected oligomycin into each of the wells, plugging the F_0 subunit of complex V, and distinguishing the contribution of ATP synthesis to the basal oxygen consumption versus the contribution of proton leak across the IMM. Next, the protonophore FCCP allowed H^+ ions to freely pass through the IMM, inducing a maximal OCR. By subtracting the baseline OCR from the maximal OCR, we can calculate the spare respiratory capacity (SRC): the reserve ATP producing potential a cell has, which is related to its number of mitochondria, the relative function of its ETC and OXPHOS components and its ability to acquire and deliver energy sources to the correct subcellular location. Following FCCP, we injected etomoxir, a CPT1 inhibitor that isolates the contribution of fatty acid versus non-fatty acid oxidation to maximal respiratory capacity. Finally, a combination of rotenone and antimycin A were added to block complexes I and III of the ETC respectively, which stops H^+ pumping to the IM space. The OCR at this point and all points past represents the non-mitochondrial oxygen consumption.

The mitochondrial flux of both PINK1 sufficient and deficient T cells activated overnight with α -CD3 and α -CD28 were statistically indistinguishable, indicating normal operation of the CD28 co-stimulatory pathway despite the loss of a protein which promotes Akt phosphorylation. Conversely, IL-2 treatment with α -CD3 in *PINK1*^{-/-} cells reveals a metabolic defect, with a clear decrease in the values of basal respiration, oxygen consumption due to ATP production, maximal respiration and spare respiratory capacity. In segment D of the analysis where FCCP allows H^+ ions to freely traverse the IMM but fatty acid transfer into the mitochondrion is blocked by the CPT-1 inhibitor etomoxir, the deficit is shown to be from non-fatty acid energy sources for OXPHOS (Figure 4.5C),

potentially related to decreased expression of the Akt-responsive gene GLUT1 (Figure 4.4E). Insufficient glucose acquisition could also be the cause of reduced basal ECAR levels seen in the same cells (figure 4.5D). Disruption of mitochondrial OXPHOS via reduced $\Delta\psi_m$ could prevent the ability of a cell to meet certain metabolic checkpoints¹⁵⁷, diverting cellular fate decisions or inhibiting effector function.

4.2.3 Induced regulatory T cell development and function requires PINK1

We and others have demonstrated the crucial role of the Akt pathway in the conversion of conventional T cells into suppressor iTregs¹⁶²⁻¹⁶⁴. Since decreased IL-2 receptor and Akt signaling oppose each other in the decision of a T cell to acquire an iTreg phenotype¹⁶⁵, we asked how *PINK1*^{-/-} T cells would react to signals from iTreg polarizing conditions. Knockout cells first exhibited a delay in the surface expression of CD25 and CTLA-4, yet never faltered in their expression of FoxP3 (Figure 4.6A). In association with this outcome, fewer *PINK1*^{-/-} cells proliferated, which is of particular interest, as the acquisition of suppressor ability is associated with multiple rounds of cell division (Figure 4.6B). When we measured the suppressor ability of sorted, PINK1 sufficient or deficient iTregs (Figure 4.6C/D), only wild type iTregs were able to dampen the proliferation of co-cultured, CFSE labeled target cells, indicating an uncoupling of FoxP3 expression and suppressor function *in vitro* (Figure 4.6E/F). The deletion of PINK1 seemed to only affect the acquisition of anti-inflammatory activity, as Th17-inducing medium could induce the simultaneous expression of both ROR γ T and IL-17 in those cells (Figure 4.6G) despite reduced GLUT1 expression (Figure 4.4E) and glycolysis rate (Figure 4.4I).

4.3 Discussion

With the recent appreciation for the effect that metabolic checkpoints and metabolic control of effector T cell development and function, the examination of T cell responses in cells lacking a key regulator of both Akt and mitophagy such as PINK1 was certainly warranted, especially as mutations in PINK1 are associated with familial, early onset PD. Here, we show that PINK1 aids in the activation of T cells and the acquisition of suppressor function in iTregs related to Akt, IL-2 and metabolism.

The dual nature of PINK1's localization suggests that the protein may play an active role in adapting a cell's response to energetic cues and TCR stimulation. As Akt was first implicated in oncogenesis, likewise has the cytoplasmic localization and increased expression of PINK1 been implicated in cancerous cells^{166,167}, an instance of uncontrolled cell division. Though we have shown above that PINK1 is important in full activation of Akt, this effect is most likely indirect, as Akt has yet to be added to the growing list of PINK1 phosphorylation targets. However, its residence in the cytoplasm and availability to influence Akt activation status relies on autophosphorylation, as kinase dead PINK1¹⁶⁸ mutants localize to the cytoplasm. PINK1 knockout T cells also show reduced the activation of mTOR as indicated by reduced phosphorylation of Akt at S473, a target of mTORC2⁴⁷, and extrapolated from the reduction of p-S308. Indeed, cells lacking PINK1 have reduced phosphorylation of ribosomal protein S6¹⁴⁹, the target of S6 kinase whose phosphorylation is frequently used to gauge mTOR function. Reduced mTOR signaling attenuates transcription and translation by modulating its targets 4E-BP1 and S6 kinase, which could explain the delayed surface expression of CD25 and CD69 reported above.

At the mitochondrion, PINK1 specifically targets mitochondria with reduced $\Delta\psi_m$ for recycling via mitophagy, increasing overall mitochondrial health. We show, in agreement with many other cell types of PINK1 deficient cells, that T cells lacking PINK1 have decreased $\Delta\psi_m$ after stimulation with α -CD3/IL-2 (Figure 4.4F). What's not clear though, is whether this decrease is related to the quiescent nature of resting cells versus their rapidly dividing, stimulated counterparts, or reflects mitophagy as an important part of the T cell activation process. Naïve CD8⁺ T cells increase their mitochondrial mass during the expansion/contraction phases of T cell activation such that memory T cells have an increased SRC dependent on IL-15 and FAO. This increased capacity for OXPHOS contributes to the increased speed of secondary activation¹⁶⁹, a hallmark of T cell memory, and demonstrates the effects of mitochondrial function on T cell phenotype. Though we did not see an increase in SRC upon activation even in wild type CD4⁺ T cells (data not shown), potentially due to our not specifically inducing T cell memory, there was a reduction in $\Delta\psi_m$ in IL-2 activated *PINK1*^{-/-} cells, which could indicate that the inability to recycle old mitochondria into new mitochondria during activation.

The PI3K/Akt/mTOR pathway engages in crosstalk with other pathways to coordinate the cellular response to changes in the extracellular environment. The function of AMP-activated protein kinase (AMPK), a negative regulator of mTOR both directly¹⁷⁰ and indirectly¹⁷¹, is modulated by fluctuations in intracellular AMP/ATP concentration or perturbations in normoxia. ATP, ADP and AMP bind to the γ regulatory subunit of AMPK to alter its activation. In cells with a high AMP/ATP ratio, and thus displaying energetic deficiency, AMPK is found in its phosphorylated, active form and vice versa.

By inhibiting mTOR at this state, AMPK is able to restrain protein synthesis in times of malnutrition or starvation. Oligomycin treatment of PINK1 knockdown cells caused a rapid decrease in $\Delta\psi_m$ ¹⁵⁹, indicating that the mitochondrial membrane potential was maintained by complex V running in reverse and hydrolyzing ATP. The increased AMP/ATP ratio and AMPK activation likely to result from this action provides an alternative, mitochondrial explanation for the PI3K/Akt/mTOR pathway inhibition seen above. An investigation into intracellular signaling in PINK1 deficient cells in response to extracellular oxygen and nutrient availability would be of interest, particularly as the AMPK activator metformin is currently prescribed to treat hyperinsulinemia, having effects on mTORC1 similar to rapamycin, which could include skewing the T cell repertoire towards iTreg development¹⁷². Following, T cells lacking AMPK have increased glycolytic levels and IFN γ production is bolstered¹⁷³.

Another activation signal downstream of TCR activation not investigated here involves the transient increase in intracellular calcium concentration¹⁷⁴. Membrane depolarization of WT neurons induces an increase in cytosolic and mitochondrial calcium. In PINK1 KO cells, this increase is accompanied by a subsequent loss of $\Delta\psi_m$ ¹⁵⁹. Notably, T cell and B cell receptor stimulation leads to a similar increase in cytosolic calcium levels which is sustained by mitochondrial action and dependent on $\Delta\psi_m$ ¹⁷⁵. It remains an interesting possibility that part of the effect of PINK1 loss is by disrupting the second messenger signaling role of Ca⁺² in its activation of NFAT, NF- κ B, CRAC channels, etc.¹⁷⁶. Since the intracellular calcium spike is differentially regulated in iTregs¹⁶², PINK1 might be necessary for T cell fate decisions downstream of mitochondrial calcium regulation.

The effect of PINK1 loss had variable effect on metabolism following stimulation with α -CD28 and IL-2 despite a deficiency in Akt phosphorylation in both pathways. Since CD28 stimulation induces IL-2 secretion, it is presumed that the IL-2 pathway is activated in cells undergoing both treatments, so that the only difference between the groups was in the IL-2 cultured cells lacking the strong stimulation of CD28 and the whole of its downstream elements¹⁷⁷. Though α -CD3 with IL-2 and TGF- β is sufficient to induce FoxP3 in wild type and PINK1 cells, levels of OXPHOS are not sufficient in PINK1 cells to endow suppressor activity, supporting the hypothesis that metabolic checkpoints control T cell differentiation.

Although others have suggested links between PINK1 and type 2 diabetes¹⁷⁸, cancer^{166,167} and psychiatric dysfunction¹⁷⁹, there exists no epidemiologic correlation between PINK1 loss and immune dysregulation, potentially related to the relative rarity of PINK1 mutations (1%-9% of early onset PD)¹⁸⁰ or to concomitant effects on pro-inflammatory cell activation *in vivo* as seen in *Raptor*^{-/-} mice²⁰. The failure of *PINK1*^{-/-} mice to develop an autoimmune syndrome could be the result of incomplete elimination of suppressor function *in vivo* or the normal development of nTregs. However, we would expect these mice to have an exacerbated reaction to a subsequent inflammatory challenge. Indeed, the effect of PINK1 loss on T cell subset differentiation via type of metabolism or mTORC2 activation^{52,148} could each skew the T cell response^{52,139}, resulting in inappropriate immune responses or autoimmunity. Particular to our own lab's interest in gut homeostasis, we would expect that these immunodysregulations to affect the microbial composition and have far reaching effects on obesity¹⁸¹ as well as peripheral¹⁸² or enteric inflammation¹⁸³.

Several studies have shown abnormalities in peripheral T cell populations in sporadic PD¹⁸⁴⁻¹⁸⁶: CD4⁺ and CD8⁺ T cell infiltrates are present in the post-mortem human Parkinsonian brain and CD4⁺ T cells directly contribute to dopaminergic neuron loss in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of PD. Yet, the low frequency of lymphocytes behind the blood-brain-barrier during non-inflammatory conditions is inconsistent with the contribution of T cells in disease initiation in PD. Rather, calcium induced cell death of dopaminergic neurons during disease¹⁵⁹ activates resident microglia to secrete cytokines, inviting T cell infiltration and potentially provoking disease progression. Mice with reduced numbers of CD4⁺ CD25⁺ Tregs showed increased microgliosis and dopaminergic neuron death in response to MPTP, whereas adoptive transfer of Tregs protected against neuron loss¹⁸⁷. Since PD is not yet curable, and prescribed drugs focus on the management of symptoms, immune system modulation could be a novel, unexplored therapeutic avenue through which disease progression can be halted. Indeed, TZD treatment blocks iNOS expression to protect from a mouse model of PD¹⁸⁸, and retinoic acid has similar iNOS-inhibiting effects in microglia¹⁸⁹. Mitochondrial dysfunction has been a focus of PD related research since the discovery that MPTP, a complex I inhibitor, caused substantia nigra cell death and PD-like symptoms in humans. Since mitochondrial dysfunction is not only present in PINK1 deficiency, but also a hallmark of all forms of sporadic PD¹⁹⁰, metabolic defects may likewise account for altered T cell subpopulations and function in sporadic PD. If so, elucidating T cell function in the absence of PINK1 may also help to unravel the role of T cells in the progression of sporadic PD. Further investigation of T cell function in carriers of PINK1 mutations may be warranted in parallel with studies in PINK1-deficient mice,

especially as mutations involving different domains of PINK1 have been discovered, and which might affect only some of PINK1's full suite of functions.

A model based on the results in this chapter can be found in Figure 4.7. During quiescence, PINK1 associates with both healthy and damaged mitochondria. By accumulating on the surface of mitochondria with low $\Delta\psi_m$, it recruits the machinery needed for mitophagy to maintain homeostatic turnover of damaged organelles. Healthy mitochondria import PINK1 for cleavage, leaving a stable, cytosolic cleavage product. Experiments re-constituting PINK1 knockout T cells with an uncleavable mutant of the protein will unravel the function of this byproduct. If the truncated PINK1 retains its ability to aid in Akt/mTOR activation and upregulation of OXPHOS, it would be interesting to hypothesize its role in signaling to the cell that mitochondria are healthy and the cell is prepared to grow and divide. Upon TCR crosslinking with CD28 and IL-2 pathway signaling, prevention of PINK1 autophosphorylation and subsequent cytoplasmic location could support the PI3K/Akt/mTOR pathway and OXPHOS to meet the cell's new energetic demands. Meanwhile, its absence from the mitochondrion would halt mitophagy and selective ETC protein turnover but not mitochondrial biogenesis, resulting in a net increase in mitochondrial mass and OXPHOS. Future studies complementing *PINK1*^{-/-} cells by transfecting expression vectors for wild type or Δ N-terminus PINK1 protein which accumulates in the cytoplasm¹⁶⁸ will aid in identifying explicitly cytoplasmic function.

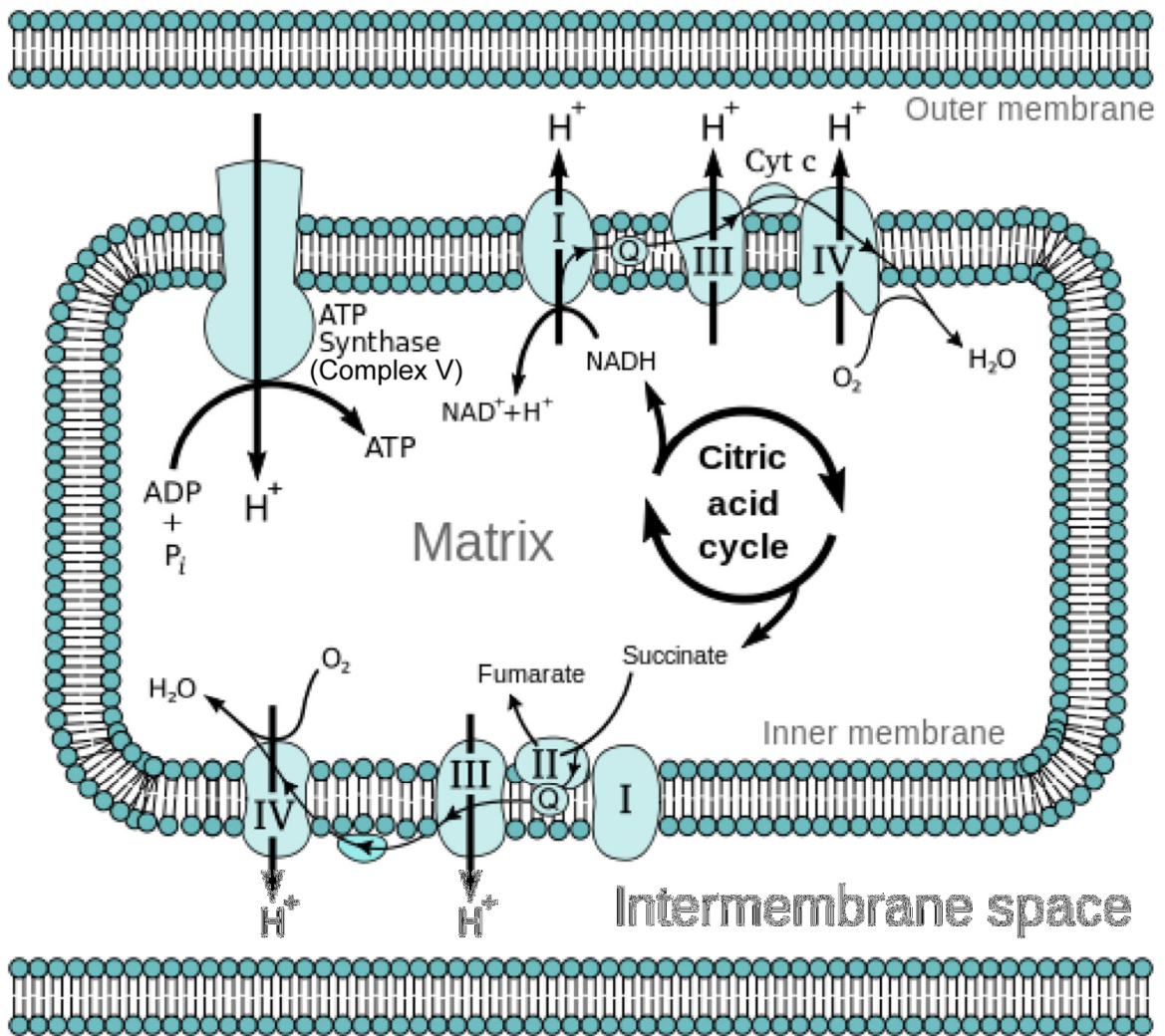


Figure 4.1 Schematic of the electron transport chain and oxidative phosphorylation. The electron transport chain drives H⁺ ions across the IM into the IM space to establish the proton motive force needed for oxidative phosphorylation at Complex V. Figure created by Dr. Timothy Vickers (Washington University in St. Louis) and released into the public domain.

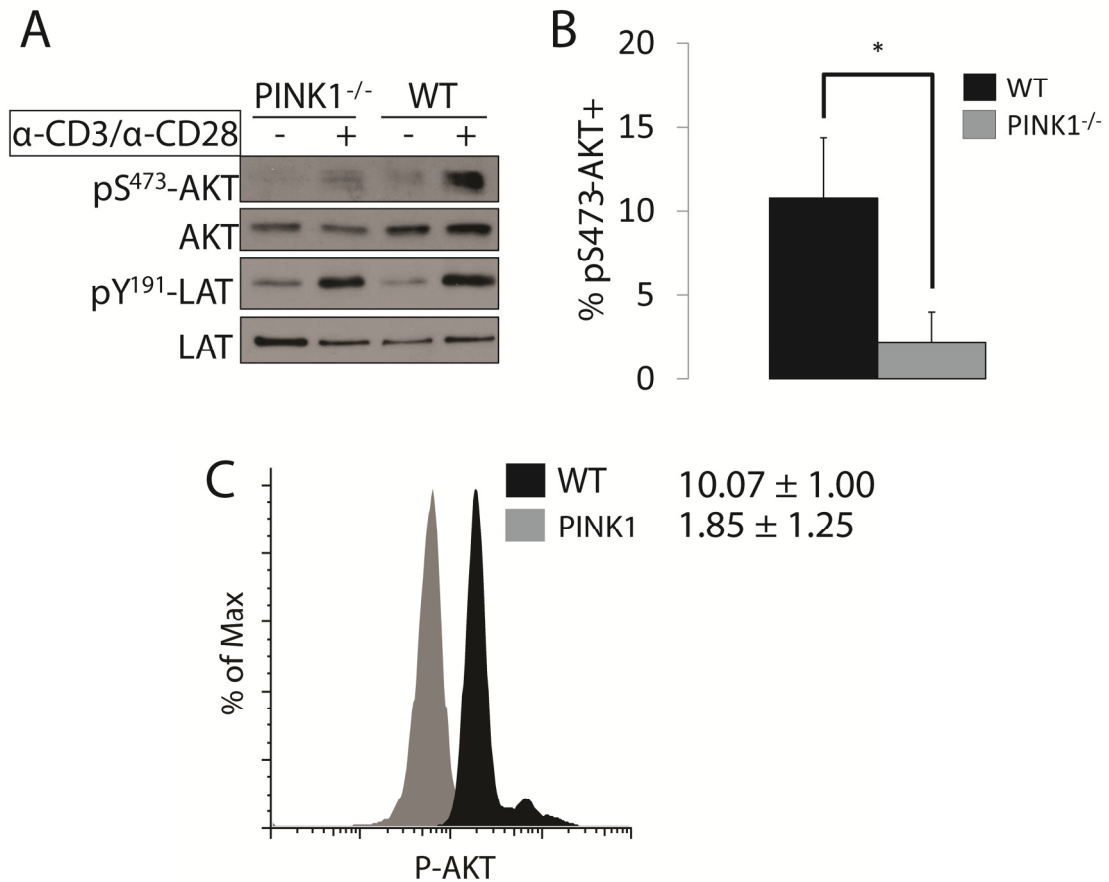


Figure 4.2 TCR crosslinking of peripheral CD4⁺ T cells requires PINK1 for optimal Akt activation. (A) Western blot comparison of activated Akt (pS473-Akt) and total Akt in peripheral LN cells after CD3/CD28 stimulation. Activated LAT (pY191-LAT) was included as a control to demonstrate equal TCR activation. (B) Percent of cells with activated Akt after stimulating peripheral LN cells for 3 min with α-CD3 and α-CD28 antibodies. Cells were first gated for lymphocytes by forward and side scatter before CD4⁺ T cells were selected. Data shown as mean + SD. (C) Representative histogram of data in B. Mean fluorescence intensity (MFI) is indicated to right of the plot. n = 4 individual mice and are representative of three independent experiments.

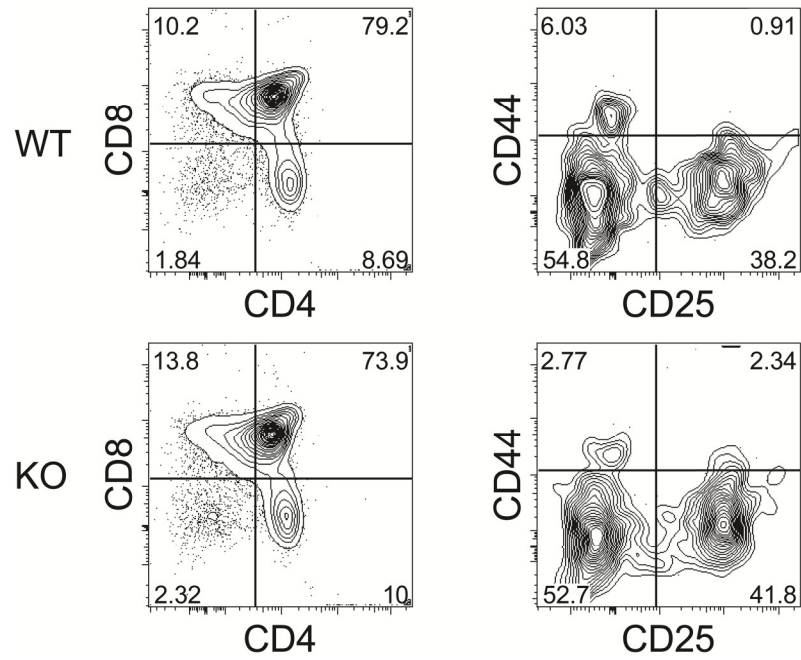
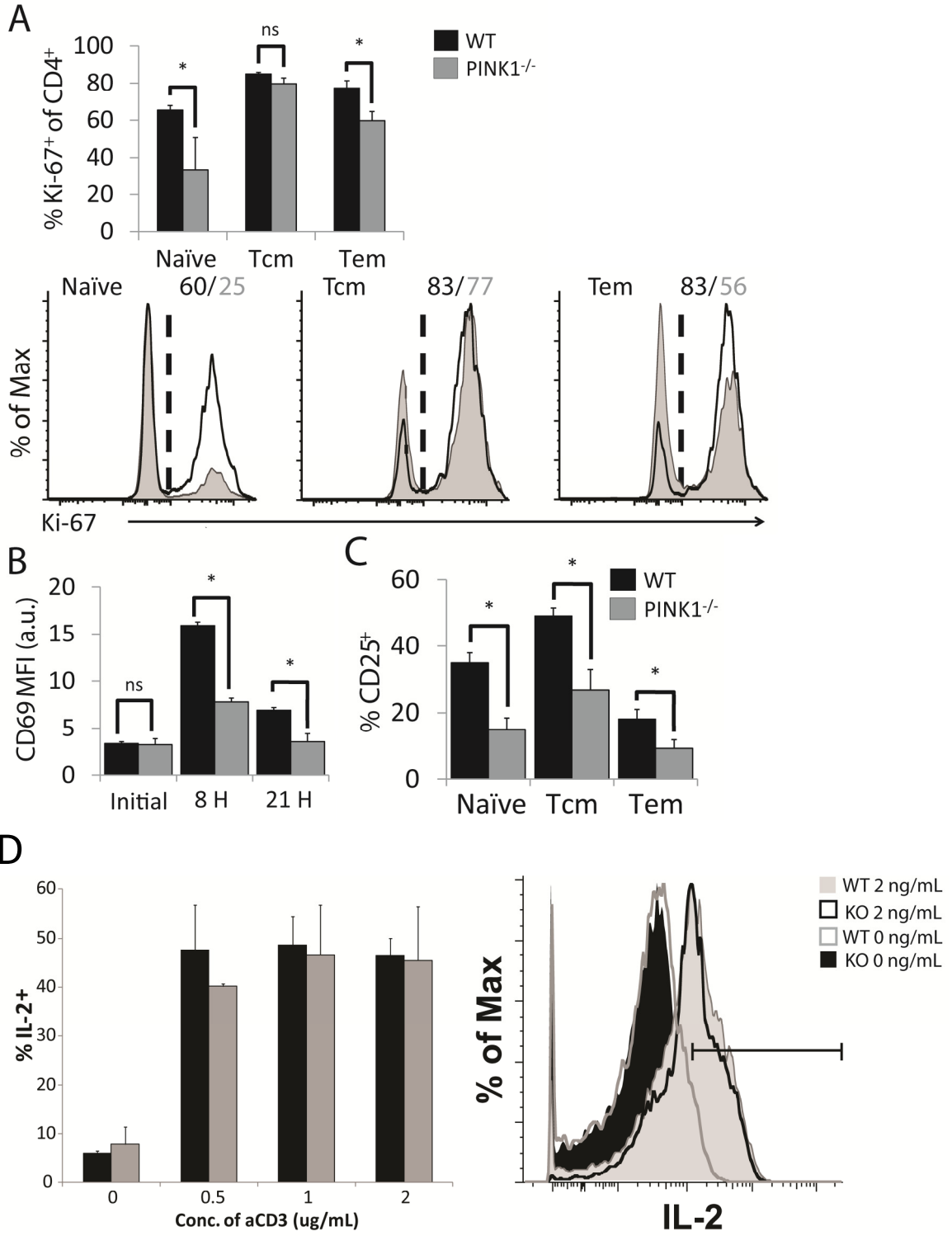


Figure 4.3 Flow cytometry analysis of thymus cells from wild type and *PINK1*^{-/-} mice. Left contour plots represent all cells, right contour plot has been gated solely on the double negative thymocytes to assess populations of DN1-4 T cell precursors, n = 3 individual mice.



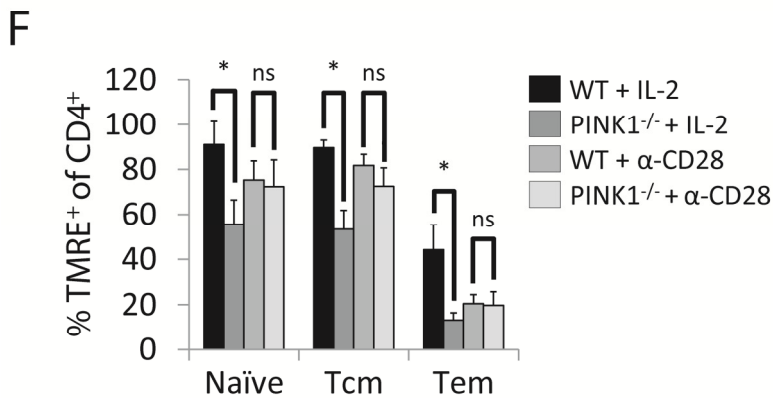
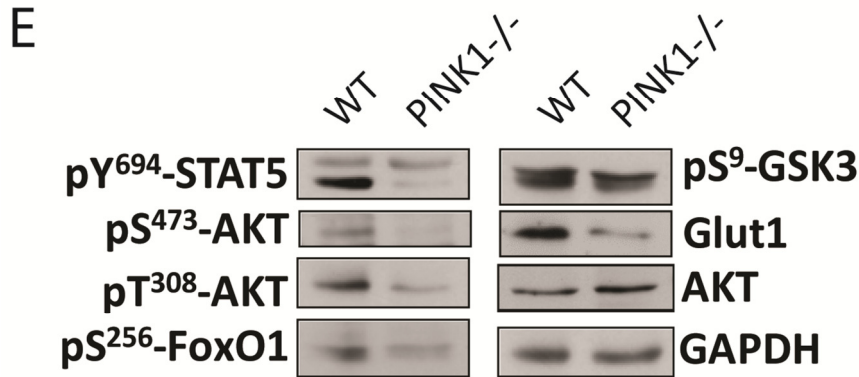
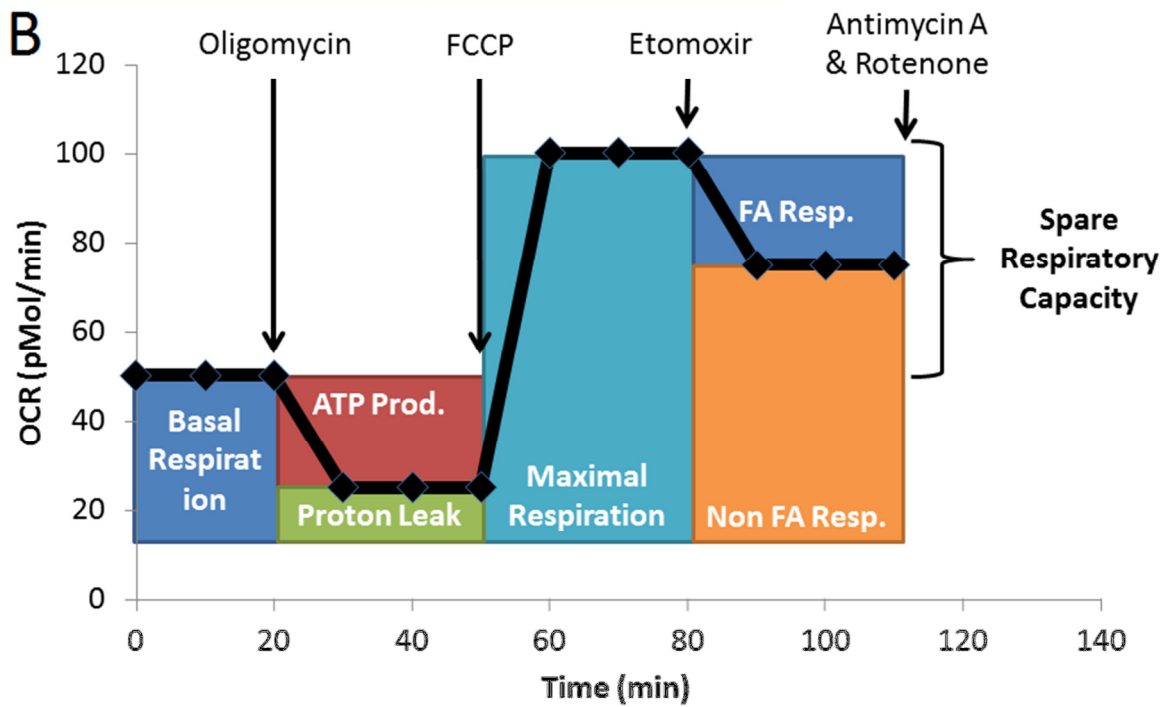
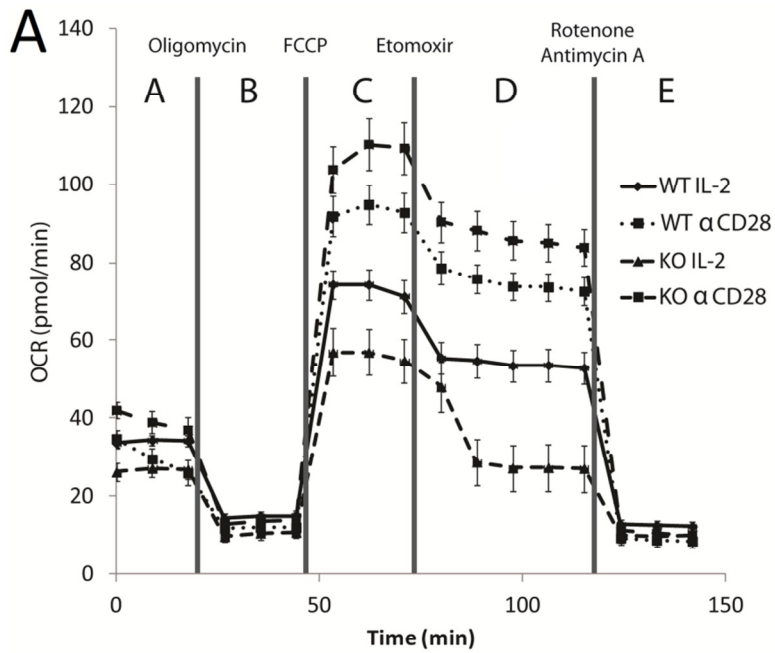


Figure 4.4 Reduced activation of CD4⁺ T cells in *PINK1*^{-/-} mice. (A) Percentages of proliferating CD4⁺ LN T cells from *PINK1*^{-/-} mice were assessed by Ki-67 expression after 48h of α-CD3/α-CD28 stimulation (left). (Naïve = CD62L⁺ CD44⁻; Tcm (central-memory T cells) = CD62L⁺ CD44⁺; Tem (effector-memory T cells) = CD62L⁻ CD44⁺) Representative histogram (right). (B) MFI of surface CD69 on CD4⁺ T cells activated as in Figure 4.4A for the indicated time points. (C) Percentage of CD25⁺ cells of CD4⁺ T cells stimulated as in Figure 4.4A. (D) Intracellular cytokine staining of T cells after 48h α-CD3/α-CD28 stimulation. Cells were then incubated with GolgiStop, PMA and ionomycin for 5 hours before flow cytometry analysis (n = 6 individual mice; mean ± SD). (E) Western blot of components of the IL-2 and Akt-signaling pathways after culture with α-CD3 and IL-2 for 48h. (F) Δψ_m was measured by flow cytometric analysis of TMRE after 48h of the indicated stimulus (n = 4 individual mice; mean ± SD). A–C, E, F: Data shown as mean ± SD pooled from at least three independent experiments, n = 6 individual mice. D: Data are representative of two independent experiments, n = 6 individual mice. *p < 0.05; two-tailed Student's t-test.



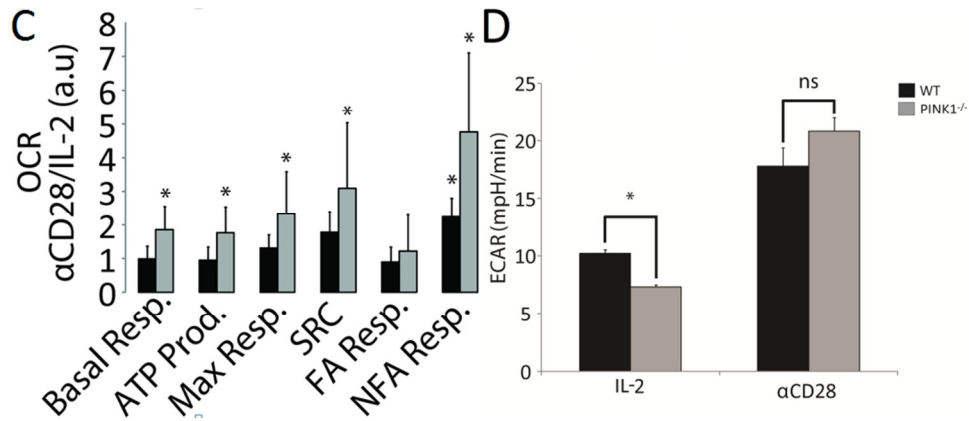
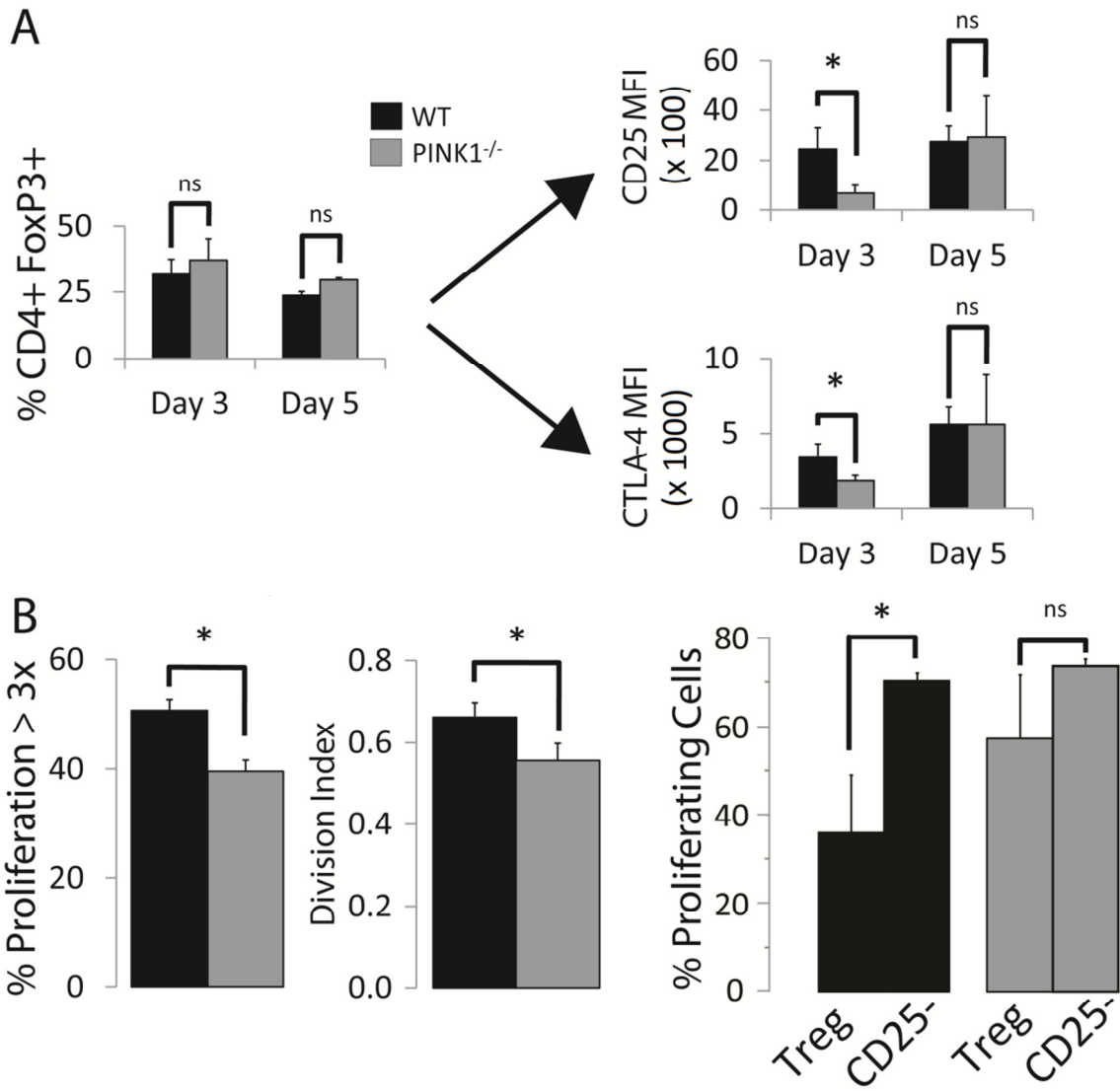
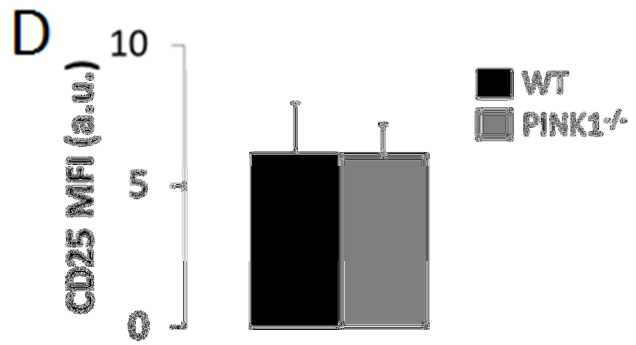
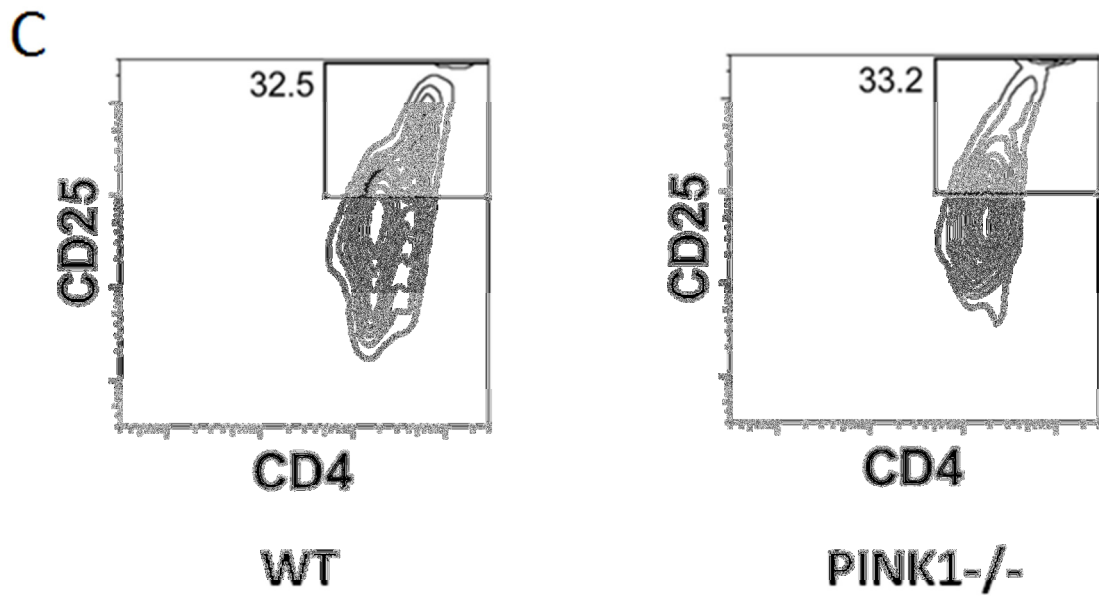
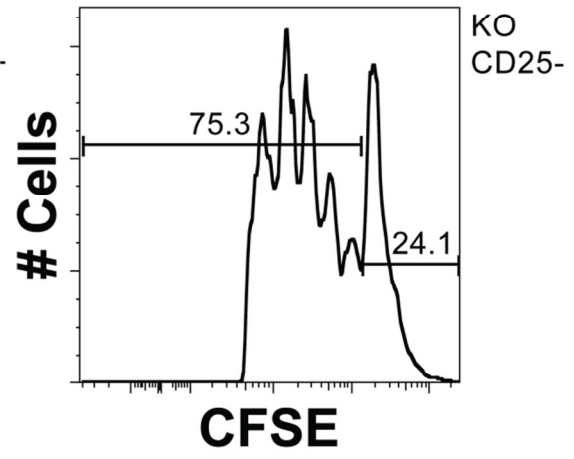
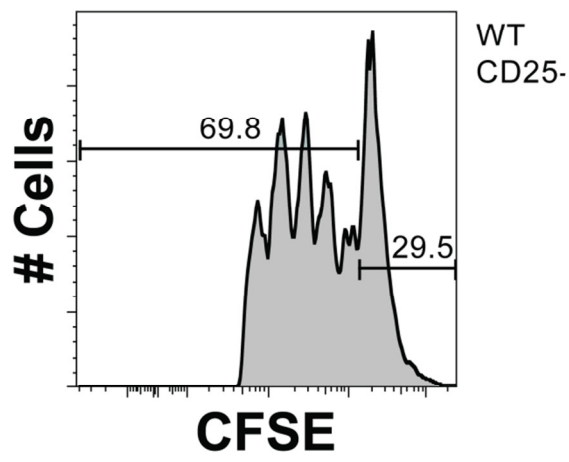
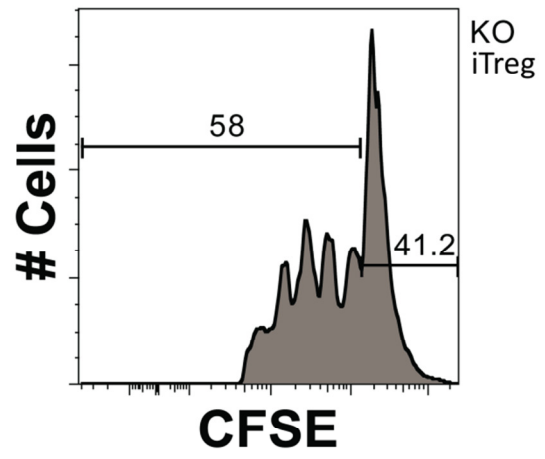
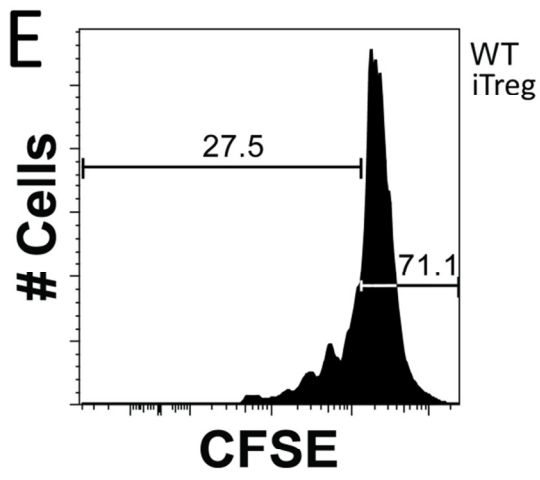


Figure 4.5 Extracellular flux analysis of activated CD4⁺ T cells reveals metabolic deficiencies downstream of IL-2 pathway (A) Mitochondrial OXPHOS was monitored by calculating the OCR via the depletion of O₂ from the surrounding medium in CD4⁺ T cells treated for 48h with either α-CD3 + IL-2 or α-CD3 + α-CD28. Oligomycin (1 μM), FCCP (1.5 μM), etomoxir (200 μM) or rotenone/antimycin A (1 μM each) were added at the time points indicated by vertical bars. (n = 4 individual mice; mean +/- SEM). (B) Derived parameters for evaluating OXPHOS are as follows: Basal resp.: A-E; ATP Prod.: A-B; max resp.: C-E; spare respiratory capacity (SRC): C-A; fatty acid (FA) resp.: C-D; Non-FA resp.: D-E. (C) Ratio of OCR between cells cultured with α-CD28 or IL-2 for 48h as a direct measure of oxidative phosphorylation, summary of experiment in 4.5B; a.u. = arbitrary units. (D) Baseline extracellular acidification rate (ECAR) from 4.5B, n = 4 individual mice; mean + SD. Data are representative of two independent experiments.







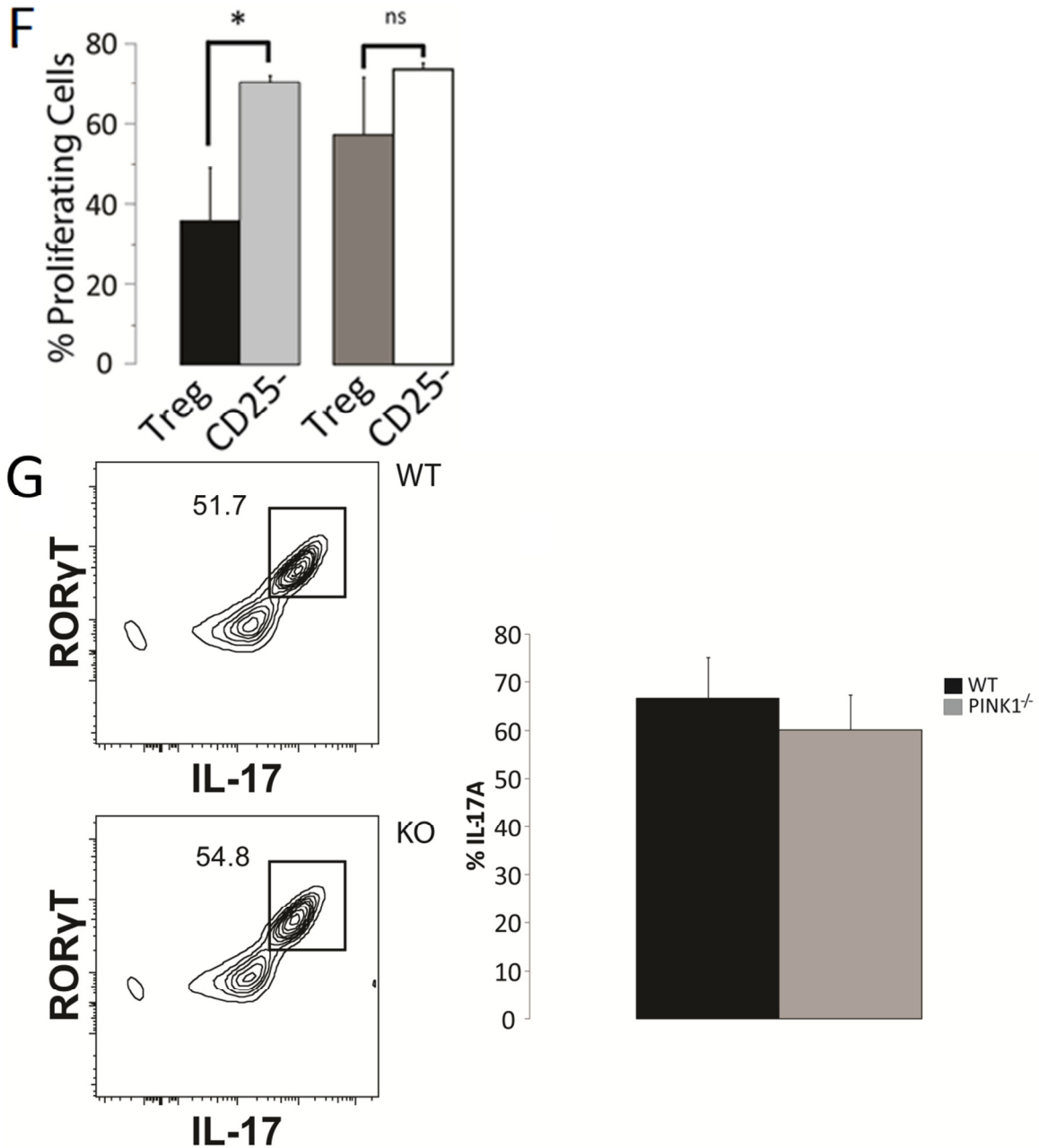


Figure 4.6 Reduced suppressor activity in *PINK1*^{-/-} iTreg cells. (A) Flow cytometric analysis of CD4⁺ FoxP3⁺ T cells and their expression of CD25 and CTLA-4 at days 3 and 5 of culture in iTreg polarizing conditions. (B) Percentage of CD4⁺ T cells having undergone more than three rounds of proliferation after culture in iTreg medium as assessed by CFSE dilution (left). Average number of divisions undergone per cell (division index) is shown in the right panel. (C) Phenotype of cells used in suppressor assay. Contour plots shapes of CD25⁺ look slightly different in wild type and *PINK1*^{-/-} CD4⁺ T cells although they show equivalent percentage of cells. (D) Sorted populations had similar mean fluorescence of CD25 (bottom) and CD25^{hi} cells. (E) CFSE labeled target cells at a ratio of 3:1 with either CD25^{hi} iTreg cells or CD25⁻ T cells were co-

cultured for 5 days with α -CD3/ α -CD28 stimulation. Representative histograms of the suppressor assay. (F) Graph representing the percentage of target cells that underwent cell division in 4.6E. Data are shown as mean + SD pooled from three independent experiments. * $p < 0.05$; two-tailed Student's t-test. (G) Representative flow cytometry plot of CD4⁺ T cells cultured in Th17 polarizing conditions for 6 days (left). Bar graph of IL-17 expression (right). Data represent mean + SD for $n = 6$ individual mice.

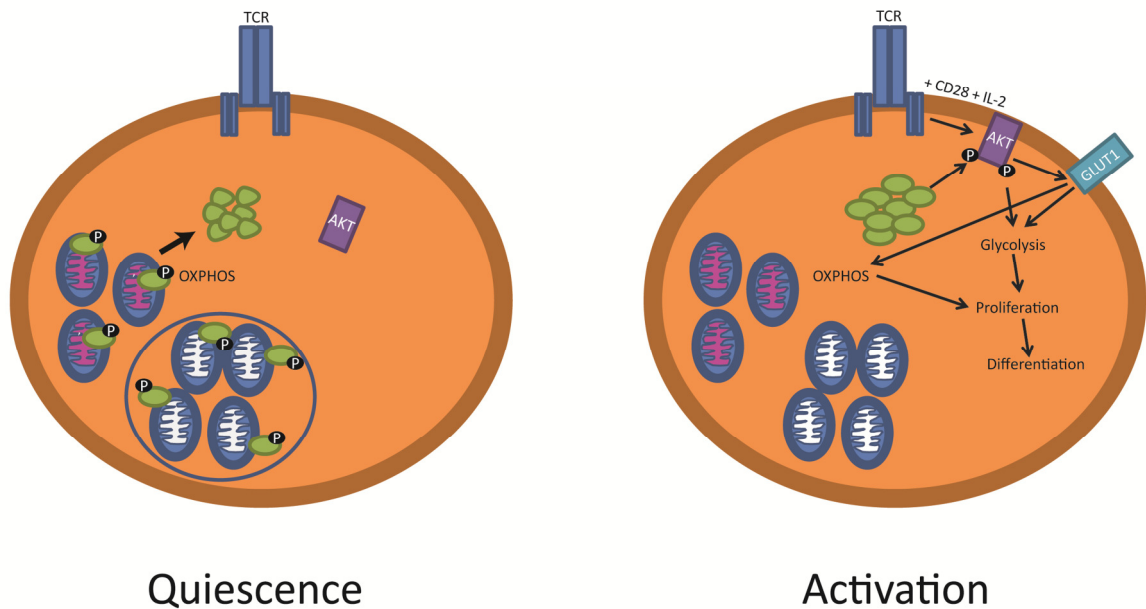


Figure 4.7 Chapter 4 model. During a quiescent state, PINK1 (green) associates with both healthy and damaged mitochondria. Healthy mitochondria import and cleave PINK1 to leave a stable cytosolic cleavage product with unknown qualities, while damaged mitochondria are destined for mitophagy to recycle cellular components. When the TCR is activated with IL-2 and CD28 stimulation, PINK1 relocates to the cytosol where it is able to enhance glycolysis and glucose uptake by fostering the activation of the Akt/mTOR pathway. Meanwhile, damaged mitochondria without PINK1 escape mitophagy.

Chapter 5: Discussion

5.1 The activities of PPAR γ /RDH10 and PINK1 are integrated through Akt/mTOR and metabolism

Over the course of this dissertation, we have shown that PPAR γ is necessary for human iTreg development, and that part of its mechanism of action is by directly binding to *RDH10* to endow T cells the ability to endogenously metabolize retinol, and that PINK1 contributes to the acquisition of suppressor activity in FoxP3⁺ iTregs by aiding in T cell activation, boosting non-fatty acid OXPHOS and maintaining healthy levels of $\Delta\psi_m$. Both the PPAR γ /RDH10 and PINK1 pathways help a T cell to sense nutrients in the environment, and are pathways integrated through the PI3K/Akt/mTOR axis and effects on mitochondrial metabolism towards subsequent iTreg differentiation and acquisition of suppressor function.

Several hypotheses exist as to the mechanism of action of retinoids in the bolstering of iTreg population which include direct antagonism of ROR γ T¹⁹¹, the enhancement of Smad3 activity¹⁹², the downregulation of the IL-6 and IL-23 receptors needed for Th17 development¹⁹², or by binding of RAR/RXR to a RARE in the CNS1 region of *Foxp3* and subsequently interfering with STAT6-mediated deacetylation¹⁹³. These mechanisms more than likely synergize as part of an ATRA program, a component of which could be through the modification of metabolism. Indeed, the treatment of isolated mitochondria with retinol is sufficient to increase OXPHOS by increasing activity of the pyruvate dehydrogenase complex which generates acetyl-CoA for the Krebs cycle¹⁹⁴ and the ATRA-induced differentiation of SH-SY5Y human neuroblastoma cells increases SRC and level of OXPHOS¹⁹⁵. Therefore, part of the iTreg boosting

mechanism of retinoids could be via the increase in OXPHOS in the presence of RAR α stimulation. Because of RDH10's localization in the MAM, we predict that its metabolism of retinol affects mitochondrial dynamics.

Similarly, PPAR γ interacts in the same arena as PINK1 via its negative regulation by Akt¹⁹⁶. As the master transcriptional regulator of adipocyte differentiation, deficiency of PPAR γ is also associated with metabolic disorders and increased free fatty acid levels¹⁹⁷, reducing their assembly into adipocytes for storage. In PINK1 knockout mice, the only overt phenotype seen is a consistent increase in body mass across both genders¹⁴⁹, indicative of a metabolic disorder, and PINK1 and PPAR γ mutations are both associated with type 2 diabetes in humans. Since PPAR γ controls the levels of unincorporated fatty acids, it regulates the ability of mitochondria to undergo FAO, critical for iTreg differentiation.

These actions of PPAR γ and retinoids integrate with PINK1 through metabolism as part of a nutrient sensing system that ensures that a differentiating T cell has the mitochondrial function and fuel sources to pass through metabolic checkpoints. Future work determining the rates of OXPHOS and glycolysis in RSG or retinoid treated T cells in wild type, PINK1 or Akt deficient mice will be of value in investigating the hypothesis that part of the iTreg-inducing mechanism of the PPAR γ and retinoid pathways is through modulating OXPHOS. In parallel, the same experiments should be performed on sorted immune cells from isolated from human disease states, especially IBD, to determine if a metabolic component of disease etiology or progression exists, identifying a population of patients which could benefit from pharmacological modulation of metabolism, including a newly-discovered PINK1 enhancer molecule¹⁹⁸.

Contemporary literature seems to be binary in its description of iTreg function (i.e. suppresses T cell proliferation and ameliorates disease, or fails to), and does very little to sort out the multitudes of potential anti-inflammatory mechanisms. In a disease state, the loss of peripheral tolerance could be attributed to (1) reduced Treg suppressor function(s), (2) fewer Tregs at site of immunopathology or (3) resistance of pro-inflammatory cells to Treg suppression. The complexity could be compounded, as disease etiology and progression can be combinatorial, synergistic and can vary between individuals despite a similar diagnosis. In chapter 4, the uncoupling of FoxP3 expression and the ability to suppress bystander T cell proliferation in FoxP3⁺ *PINK1*^{-/-} Tregs should not represent a complete failure of suppressor ability, but rather indicates that the entire suite of suppressor mechanisms under the transcriptional program of FoxP3 has not been engaged as a result of PI3K/Akt/mTOR signaling and metabolic deficiencies. iTreg function in other *in vitro* suppressor assays (e.g. ability to suppress DC activation) or *in vivo* models of autoimmunity could remain fully operational to wild type levels, and such experiments are needed to fully characterize Akt dependent versus independent suppressor functions of iTregs.

5.2 Overall significance

The fundamental impetus for conducting the research presented here is to improve our understanding of human regulatory T cell biology, so that it may be translated into novel therapeutics for pathology related to immune dysfunction. Though modulation of iTreg populations has shown prophylactic and therapeutic efficacy in murine models of immunopathology and autoimmunity, the translation of these protocols to the clinic has

not yet been as successful because of our incomplete understanding of human iTreg biology.

Two strategies for iTreg therapy might be used, either *ex vivo* or *in vivo* treatment, each with its own benefits and drawbacks. With *ex vivo* therapy, a patient's blood is drawn, T cells are isolated, cultured in appropriate medium, sorted and re-infused. Notwithstanding the high cost of such a procedure (see: autologous dendritic cell vaccines), we as of yet do not have a panel of cell surface markers which can definitively identify a pure population of suppressor T cells. Though FoxP3 does identify pure Tregs in murine populations, activated effector T cells in humans transiently upregulate FoxP3, and because FoxP3 is an intracellular protein, its detection requires permeabilization of a cell, resulting in its death. *Ex vivo* handling also requires a strict chain of custody to ensure a patient receives his own blood product, sufficient sterility and good manufacturing practices. The re-infusion of pro-inflammatory effector cells even at scarce levels could amplify the immune response and exacerbate disease. Handling of the cells does provide the opportunity for genetic manipulation – perhaps to correct a defective, disease-causing pathway, or inserting a suicide vector which instructs cells to apoptose upon FoxP3 withdrawal – and avoids the side effects of systemic drug administration. *in vivo* treatment involves oral, injectable or suppository drugs directly into the body. Its most infamous application occurred in the trial of TGN1412, a superagonist antibody directed against the costimulation receptor CD28. Though efficacious in rodent and simian trials, injection of TGN1412 into human recipients could not target Treg populations with any specificity, and thus rapidly expanded pro-inflammatory T cells into a near-fatal cytokine storm¹⁹⁹. The advantage of *in vivo*

treatment though, is the ease at which a patient can self-medicate, practically able to stay on one set of medications for perpetuity. Regardless of whichever therapeutic modality emerges as the clinician's choice, the control of Treg function must be impeccably tuned, as an over-exuberant response will reduce peripheral immunosurveillance and result in an immunodeficient state. Successful pathogens and tumors have been known to harness this facet of Treg biology via manipulation of their own microenvironments with the secretion of TGF- β , retinoic acid or other pro-Treg molecules.

In the pro-inflammatory context of gut inflammation, PPAR γ stimulation is part of the mechanism of action of the oft-prescribed IBD drug 5-aminosalicylic acid (5-ASA)²⁰⁰, and high vitamin A therapy also has anti-colitic effects¹⁰⁰. Based on our work in chapter 3, we believe that these pathways are connected through RDH10 activation and in dendritic and T cells, and that this link can be exploited therapeutically. The supplementation of the diet with oral retinyl esters would boost the efficacy of 5-ASA via its iTreg inducing effects. Since the majority of T cells in the pathogenic and non-pathogenic gut are effector and memory T cells that remain inert towards ATRA, PPAR γ stimulation has the potential to upregulate RDH10 and RAR α to enable ATRA responsiveness. In addition, modulation of specific mTORCs could act in the specific suppression of Th1 responses in Crohn's disease or Th2 immunity in ulcerative colitis.

5.3 Conclusion

The work described here highlights the PI3K/Akt/mTOR pathway as a molecular hub for the integration of extracellular signals and ensuing T cell fate decisions in the control of iTreg differentiation and function. As the acquisition of iTreg phenotype occurs in a two-step process – cells undergo an initial proliferative burst followed by a

period of differentiation – the kinetics of molecular signaling must differ to support each phase, which could be regulated by Akt and PPAR γ . In the initial 24 hours after TCR stimulation, the PI3K/Akt/mTOR pathway is very active, executing the switch from OXPHOS to glycolysis, rapidly increasing cell growth and producing biomolecules for new cell synthesis. Although RAR α and PPAR γ ligands could be available to the cell, active Akt suppresses RAR (via direct phosphorylation²⁰¹) and PPAR γ (via phosphorylation of its co-activator PGC- α ¹⁹⁶). Following the ensuing cellular divisions, TCR stimulation is detrimental to iTreg differentiation and expression of FoxP3; T cells stimulated *in vitro* for 54 hours do not express FoxP3, while those that had TCR stimulation withdrawn after 18 hours see a robust upregulation^{165,202}. Following, the addition of PI3K, Akt or mTOR antagonist to the culture medium during the later 36 hours greatly increases iTreg differentiation, mimicking the cessation of CD3 and CD28 signaling¹⁶⁵. The later release of Akt from activation could not only catalyze the switch back to OXPHOS, but would also derepress RAR α and PPAR γ , allowing for their pro-iTreg signals in addition to the maintenance of high levels of OXPHOS.

We also confirm in a second system (Raptor knockout being the first²⁰) that FoxP3 expression in the absence of functional Akt/mTOR and mitochondrial ATP metabolism is not sufficient to endow suppressor function in murine Tregs, where FoxP3 was thought to be sufficient. Upon TCR stimulation, PINK1 is involved in the phosphorylation of Akt, increasing the uptake of glucose, early T cell activation, OXPHOS and glycolysis, allowing the developing T cell to reach molecular checkpoints that regulate the immune response. Though an increase in OXPHOS is part of the iTreg gene regulatory program downstream of FoxP3, interfering mutations downstream of

FoxP3 or the migration of a T cell to a hypoxic or nutrient poor microenvironment could prevent the acquisition of suppressor function. Though this metabolic checkpoint provides an additional regulatory mechanism to control T cell function, its dysregulation represents a further source for immunological dysregulation. Overall, our work provides a new molecular framework on which to monitor for deficiencies in autoimmune disease, and to base new therapeutics for the creation of stable, effective, properly trafficked iTregs in IBD or PD.

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