12-18-2020

Liver Kinase B1 isoform expression as a regulator of T cell phenotypic stability

Heather Sherman

Liver Kinase B1 isoform expression as a regulator of T cell phenotypic stability

A Dissertation Presented

by

HEATHER L SHERMAN

Submitted to the Graduate School of the
University of Massachusetts, Amherst in partial fulfillment
of the requirements for the degree of

DOCTOR OF PHILOSOPHY

September 2020

Program in Molecular and Cellular Biology

Liver Kinase B1 isoform expression as a regulatory of T cell phenotypic stability

A Dissertation Presented

by

HEATHER L SHERMAN

Approved as to style and content by:

_____
Lisa M. Minter, Chair

_____
Barbara A. Osborne, Member

_____
Wilmore C. Webley, Member

_____
Pablo Visconti, Member

_____
Scott C. Garman, Director
Program in Molecular and Cellular Biology

DEDICATION

To my late grandfather, Grandpa Joe who always believed in all I did and was so happy that I wanted to pursue a research career in science.

ACKNOWLEDGEMENTS

I would like to thank Dr. Lisa M. Minter for all her support and guidance during my graduate work. Throughout my work, Lisa has allowed me the freedom to explore different avenues while also always being there to bring me back before I deviated too far off course. This has allowed me to grow as a scientist, tremendously. Lisa is incredibly humble, thoughtful, and caring. She is the kind of scientist that I hope to exemplify in my work. I cannot thank her enough for giving me the opportunity to perform my dissertation work in her lab, putting up with my crazy ideas, and being there not only as a mentor but a friend. I will truly miss our hour-long meetings where science was only the beginning. Dr. Barbara A. Osborne has been an incredible mentor through this whole process. From bonding over being Jersey girls to intense scientific discussions, Barbara has motivated me to think outside the box and never think a hypothesis is too crazy…until you test it. Dr. Wilmore Webley has provided great insight into this work and has been a great resource. I am especially grateful for the teaching experience he provided me in his immunology lab course. Dr. Pablo Visconti has provided great support and guidance in my dissertation work. Pablo taught me how to write my first grant and always challenged me to think critically about my work and other's work.

I would also like to thank Dr. Amy Burnside who always saved the day when instruments were acting up or if I had questions about data analysis. I have really enjoyed working with her and will very much miss our chats. Thank you to Dr. Cynthia Baldwin who has always made me feel at home in the department. I also had the

pleasure of being a TA for her course, in which she allowed me to generate some of the course materials, which was a wonderful experience.

I would like to thank my Osminter Lab family: Emrah Ilker Ozay, Daniel Garrigan, Karthik Chandiran, Joe Torres, Bhaskar Reddy, Hosanna Kim, Deeksha Mohan, Rebecca Lawlor, Sudarvili Shantalingam, Ankita Mitra, Jyothi Vijayaraghavan, Mine Canacki, and Khushboo Singh. I have so enjoyed having joint lab meetings where we would chat for hours about science and life, in addition to our many lab outings and get togethers. Thank you for all your scientific and emotional support, as well as your friendship.

I would like to thank all my friends who have supported me on this journey. To Gavriella Hecht, thank you for always believing in me and knowing I could achieve this even if I did not believe it. Thank you to Constance Angelou for your support, and guidance, and for always challenging me to be a better version of myself. Thank you to my Chabad family for always being there throughout my time at school. Their warmth, support, and guidance helped me feel at home in Amherst. To Amanda Alvarez, thank you for your continuous friendship and support, even when living very far from each other. To my dear friend Rachel Weisman, thank you for your friendship, guidance, patience, and support.

To my family, thank you for your endless support. My parents, Samuel and Michele Sherman and my brother, Matthew, have always been there for me and guided me no matter what I wanted to do. I could not have done this without them.

ABSTRACT

LIVER KINASE B1 ISOFORM EXPRESSION AS A REGULATOR OF T CELL PHENOTYPIC STABILITY

SEPTEMBER 2020

HEATHER L SHERMAN, B.S., STATE UNIVERSITY OF NEW YORK AT BINGHAMTON

Ph.D., UNIVERSITY OF MASSACHUSETTS, AMHERST

Directed by: Dr. Lisa M. Minter

Following activation, CD4 T cells undergo extensive metabolic and transcriptional changes to respond to external cues and differentiate into T helper (Th) cell subsets. This differentiation process was originally perceived to be terminal. However, it has been shown that T cells exhibit plasticity between subsets in the context of highly inflammatory environments. This phenomenon is especially prevalent in autoimmune conditions such as colitis and multiple sclerosis in which high levels of IL-6 promote considerable plasticity between regulatory T (Treg) cells and Th17 cells. Herein we show that Liver Kinase B1 (LKB1), a metabolic sensor that enforces energy-preserving mechanisms such as fatty acid oxidation (FAO), is spliced into its short isoform in Th17 cells but not in induced (i)Tregs. We demonstrate that, in Th17 cells, heterogeneous nuclear ribonucleoprotein L-like (hnRNPLL) binds to transcripts of *Stk11*, which encodes LKB1, and this correlates with alternative splicing into its short splice variant (*Stk11*$_S$).When we neutralize hnRNPLL function using a cell-penetrating antibody, we observe diminished *Stk11*$_S$ expression. We further show that hnRNPLL and *Stk11*$_S$, both, are regulated by the T cell-specific kinase, Protein Kinase C theta (PKCθ) in Th17 cells. We provide additional evidence that, in iTregs exposed to IL-6, *Prkcq, Stk11*$_S$, and *Rorc* are all upregulated, suggesting iTreg-Th17 plasticity is

induced in response to IL-6 and culminates in *Stk11* splicing downstream of PKCθ. Finally, we demonstrate a link between *Stk11* splicing and Th17 metabolism, showing that functionally inhibiting hnRNPLL modulates expression of the key glycolytic enzyme, Hexokinase 2, and inhibiting glycolysis, in turn, modulates the expression of $Stk11_S$. Our data reveal an as-yet-undescribed outside-in signaling pathway downstream of IL-6 that acts through PKCθ and hnRNPLL to regulate splice variants of *Stk11* in Th17 cells. Furthermore, we show for the first time that this pathway can also be initiated in iTregs exposed to IL-6, providing mechanistic insight into iTreg-Th17 plasticity.

TABLE OF CONTENTS

Page

LIST OF TABLES

Table Page

LIST OF FIGURES

Figures Page

CHAPTER 1

INTRODUCTION

1.1 T cell activation and differentiation

T cells are activated by antigen encounter and differentiate to illicit various effector responses. The details of T cell activation, differentiation, and the regulation of T cell responses by regulatory T cells will be discussed herein.

1.1.1 T cell activation

Mature naïve T cells circulate through secondary lymphoid organs where they encounter antigen presenting cells (APC). To maintain the naïve state without cell death, the cell requires tonic T cell receptor (TCR) signaling and IL-7 stimulation (Chapman et al., 2019). The APC expresses major histocompatibility complex (MHC) proteins loaded with peptide from self-tissue or pathogens. T cells will become activated through engagement of the peptide:MHC complex with the T cell receptor which is associated with CD3 (Hoefig and Heissmeyer, 2018). To achieve sustained activation, proliferation and subsequent differentiation, T cells need to receive two additional signals. The second signal is initiated through the interaction of CD28, on the T cell membrane, and CD80 or CD86, on the APC surface. CD28 stimulation is crucial to prevent the onset of anergy and for the activation of signaling cascades modulated by kinases such as Protein Kinase Cθ (PKCθ; Berg-Brown et al., 2004).

The interactions between TCR:MHC and CD28:CD80/86 will promote the formation of an immunological synapse between the T cell and APC. The synapse requires rearrangement of the T cell cytoskeleton and movement of the lipid rafts to the point of contact. Lipid rafts are lipid microdomains rich in glycosphingolipids that act as docking sites for signaling proteins (Lucas et al., 2004). Several signaling cascades are activated downstream of CD3 and CD28 that culminate in the activation of the NF-κB, AP-1, and NFAT transcription factors which are necessary for complete T cell activation. These transcription factors will drive the expression of IL-2, a T cell survival and growth factor, (Hoefig and Heissmeyer, 2018). In addition to CD3 and CD28 stimulation, T cells require cytokine signaling to sustain their survival, proliferation, and aid in their differentiation into effector cells (Zhou et al., 2009). Figure 1 illustrates the three signals needed for complete T cell activation.

1.1.2 Cytokine signaling

Cytokines signal through cognate receptors which is bound intracellularly to Janus Kinase (JAK). JAK is activated by ligand binding and drives JAK tyrosine autophosphorylation and phosphorylation of the intracellular domain of the cytokine receptor. This tyrosine phosphorylation creates a docking domain for signal transducers and activators of transcription (STAT) proteins to bind to the cytokine receptor and, thus, allows JAK kinases to phosphorylate STAT proteins. Tyrosine phosphorylation creates a SH2 domain on the STAT protein, which allows it to dimerize with other STATS. Once dimerized, STAT proteins can translocate to the nucleus and bind gene promoters to complete the cytokine signal (Leonard and O'Shea, 1998).

There are 4 different JAK kinases and 7 different STAT proteins which can be combined to elicit different functional outcomes, specific to the cytokine receptor signal propagation (Dodington et al., 2018). Specificity of the cytokine signaling is mediated by unique STAT protein dimers which dictate downstream gene expression, and activation of specific T cell effector programs (O'Shea and Plenge, 2012). A schematic of JAK/STAT signaling is illustrated in Figure 2.

1.1.3 CD4 T cell differentiation

Activated CD4 T cells differentiate into helper T cells to elicit specific immune responses to different types of pathogens (Zhu et al, 2018). There are several different helper T cell subsets; the main subsets are depicted in Figure 1.3. Cytokines produced by APCs signal to the newly stimulated T cell to influence which helper T cell subset program is activated. Specific cytokines, signaling through JAK/STAT, upregulate signature transcription factors which are required for effector functions characteristic of each helper subset (Schmitt and Ueno, 2015; Zhu et al, 2018).

The T helper (Th) 17 cell program is initiated by IL-6 signaling through the IL-6 receptor and subsequent activation of JAK1 and STAT3. IL-6 is an 80kDa protein produced by APCs, endothelial cells, and monocytes (Schaper and Rose-John, 2015). IL-6 is a pleiotrophic cytokine that can signal to induce different processes ranging from inflammation to bone remodeling (Tanaka et al., 2014). The IL-6 receptor (IL-6R) is a type I transmembrane receptor found on immune cells, such as CD4 T cells and monocytes. Upon ligand binding, the IL-6R associates with a longer transmembrane protein, gp130, which is constitutively bound to a JAK protein. Once gp130 and the IL-6R bind, JAK proteins are autophosphorylated, leading to subsequent STAT3 activation (Schaper and

Rose-John, 2015). STAT3 dimers will bind to the *Rorc* and *Il17* promoters inducing the effector functions of Th17 cells. The Th17 program also needs activation through transforming growth factor beta (TGF-β; Zhu et al, 2017). TGF-β will also induce the activation of RORγT to further perpetuate IL-17 production and stability of the Th17 phenotype (McGeachy and Cua, 2008).

Th17 cells secrete IL-17 to primarily combat extracellular bacteria which are found at mucosal surfaces (Ivanov et al., 2006; Zhu, 2018). IL-17 will promote more Th17 cell polarization, B cell responses, induce the production of pro-inflammatory chemokines to recruit neutrophils to the site of infection, and program the macrophage response. Additionally, the pro-inflammatory profile induced by IL-17 signaling will propagate an inflammatory response through non-immune cells such as endothelial and epithelial cells which can secrete antimicrobial peptides (Iwakura et al., 2011). Th17 cells are thus the orchestrators of immunity within the mucosal compartment.

1.1.4 Thymic and peripheral regulatory T cell development

Regulatory T cells (Treg) are crucial for limiting the extent of an immune response and preventing immune attack on self-tissue. Naturally occurring Tregs (nTregs) arise from the thymus or can be induced in the periphery (iTregs). Tregs suppress other immune cells through a variety of mechanisms mediated by the signature transcription factor, forkhead box p3 (Foxp3; Deng et al., 2019; Kitagawa and Sakaguchi, 2017). Foxp3 mutations lead to the immune dysregulation, polyendocrinopathy, enteropathy, X-linked (ipex) syndrome which causes widespread autoimmunity due to Treg deficiency. Foxp3 expression can be induced by Foxp3, itself, or by GATA-3 or STAT5 (Ohkura et al., 2012). Foxp3 expression is regulated at its promoter through three conserved, noncoding

sequence (CNS) regions (Kanamori et al., 2016). CNS2, also known as the Treg cell-specific demethylated region, or TSDR, must be demethylated to sustain Foxp3 expression levels (Ferreira et al., 2019). Prior to antigenic stimulation, Treg cells are characterized as central Treg cells which express the chemokine receptor, CCR7, and CD62L which allows for tissue migration. Treg cells receive antigen stimulation as effector T cells do in the context of peptide:MHC and CD28 stimulation. Upon stimulation, Treg cells become effector Tregs which downregulate CCR7 and CD62L and subsequently upregulate CD44 (Rothstein and Camirand, 2015).

Naturally occurring Tregs develop in the thymus during negative selection when single-positive CD4 thymocytes are challenged with antigens expressed by the transcription factor *Aire*. If the thymocytes are highly reactive to any self-antigen, the cell will undergo apoptosis. A moderate reaction to self-antigen induces the upregulation of CD25 and Foxp3 (Owen et al., 2019). These cells will exit to the periphery and circulate until they are activated (Rothstein and Camirand, 2015). Figure 1.4 illustrates the generation of nTregs.

iTreg cells are generated by TGF-β and IL-2 signaling, and can be induced both *in vivo* and *in vitro.* There are specific organs, such as the intestines and the placenta, which are highly enriched in iTreg cells that suppress immune responses against food antigens and which mediate fetal rejection, respectively. The upregulation of Foxp3 in iTreg cells is mediated by TGF-β signaling to stimulate the CNS1 region. Whereas IL-2 signaling in these cells will upregulate CD25 which is necessary Treg survival and suppressive function (Kanamori et al., 2016).

1.1.5 Treg-mediated suppression

Treg cells can suppress both APCs and effector T cells using several different mechanisms. The preference for suppressing one cell type over another seems to be tissue- and target cell-dependent. In addition, Tregs can upregulate cytokine receptors to make them more suppressive against specific cell types (Duhen et al., 2012). One such suppressive mechanism is infectious tolerance which is mediated by transferring suppressive functions from Tregs to another cell type. The most conventional form of this type of suppression, is through the release of anti-inflammatory cytokines (Ferreira et al., 2019). It was originally thought that Treg cells only secrete the cytokine, IL-10; however there is emerging evidence that Tregs can also secrete the anti-inflammatory cytokine, IL-35 (Shevach, 2009). Signaling through the IL-10 receptor activates TYK2, a JAK kinase, which further activates SHP-1, a phosphatase, inhibiting processes downstream of CD28 ligation (Boonpiyathad et al., 2019). Another mechanism by which Tregs can inhibit effector T cells is by acting as a sink for IL-2 in the surrounding environment. This leaves less IL-2 available for effector T cells and in the absence of sufficient IL-2 signaling, effector T cells will undergo apoptosis. Tregs can also suppress effector T cells *via* direct lysis of the cell through the actions of Granzyme A or B. However, it is not clear whether APCs or effector T cells, or both are lysed by Treg cells, nor is it clear through which enzyme, Treg-mediated cytolysis occurs (Shevach, 2009).

Treg cells also express immune checkpoint molecules such as cytotoxic T-lymphocyte associated protein-4 (CTLA-4), Programmed Cell Death-1 (PD-1), lymphocyte activating gene-3 (LAG3), and T cell immunoglobulin and immune receptor tyrosine-based inhibitory motif domain (TIGIT). CTLA-4 is a homologue of CD28 and can

engage CD80 or CD86 on APCs. These interactions can affect the APC in two ways: creating a physical barrier to CD28 ligation on the T cells and causing downregulation CD80 and CD86 on the APC. In the absence of CD80 or CD86 expression, the APC can still engage the TCR on effector T cells, but without CD28 ligation the T cell will undergo anergy. CTLA-4 engagement with CD80/CD86 can also induce the APC to secrete indoleamine 2,3-dioxygenase (IDO) which converts tryptophan into pro-apoptotic proteins, thus killing surrounding effector T cells (Shevach, 2009). Tregs also suppress through Programmed Cell Death Protein-1 (PD-1) receptor interaction with its two ligands, Programmed Death Ligand 1 and 2 (PD-L1 and PD-L2). PD-1, on the Treg, will bind its cognate ligand on the APC, inducing APC cell death. Additionally, PD-1 on the Treg further enhances Foxp3 expression and the subsequent effector function of the Tregs (Gianchecchi and Fierabracci, 2018). LAG3 is a homolog of CD4 that is thought to bind MHC class II proteins on dendritic cells. LAG3-MHC class II binding blocks dendritic cell maturation, making them unavailable to promote T cell activation (Shevach, 2009). TIGIT can also be expressed on Tregs and competes with CD226 to bind CD155 on dendritic cells. Engagement of TIGIT with CD155 inhibits dendritic cell maturation (Joller et al., 2014; Kurtulus et al. 2015). TIGIT-expressing Tregs are highly suppressive, express higher levels of Foxp3, PD-1, CTLA-4, and secrete more IL-10 (Kurtulus et al., 2015). Interestingly, these Tregs are autophagy-dependent which has been shown to be important in Treg survival (Le Texier et al., 2016).

1.2 T cell metabolism

T cells will undergo metabolic changes during its lifetime to accommodate for its energetic demands. Additionally, different subsets of T cells can engage different

mechanisms of energy production but tend to rely more heavily on one metabolic pathway. Ultimately the goal is to produce acetyl-coA to fuel the Citric Acid Cycle (TCA) and electron carriers for oxidative phosphorylation (OXPHOS) to produce ATP. This can be done through several processes, as shown in Figure 1.5 (Galgani et al., 2015). In the next section, preferences in energy metabolism for naïve, effector, and regulatory T cells will be discussed.

1.2.1 Metabolism of the naïve T cell

Naïve T cells are metabolically quiescent until they encounter antigen. During their quiescence, T cells favor energy-preserving metabolic processes. Instead of importing glucose or amino acids the cell will breakdown intracellular stores of these nutrients to fuel mitochondrial ATP production through oxidative phosphorylation (OXPHOS) and the tricarboxylic acid (TCA) cycle. It is crucial to maintain IL-7 signaling during quiescence, as IL-7 promotes the breakdown of necessary molecules to fuel OXPHOS (Chapman et al., 2019). After glycolysis, pyruvate is further modified into acetyl-coA by pyruvate dehydrogenase. Acetyl-coA will be fed into the TCA cycle to generate the electron carriers FADH2 and NADH. These electron carriers are used for OXPHOS to generate ATP (Lochner et al., 2015; Ma et al., 2017; Galgani et al., 2015; Blagih et al., 2012).

1.2.2 Metabolic changes in the activated T cell

Upon activation, naïve T cells undergo a process known as the “Warburg Effect”, depicted in Figure 1.6, which changes the cell’s primary means of energy production from catabolism to anabolism (van der Windt and Pearce, 2012). T cells experience various changes after activation to accommodate for increases in cell size, proliferation, and

acquisition of effector functions. These changes require increased energy production and the accumulation of biomass. A naïve T cell can break down its cellular components to feed OXPHOS; however, this is a slow process and is not sufficient to fuel an effector cell's new energy requirements (Blagih et al., 2012).

The Warburg Effect allows cells to use glycolysis to convert glucose into lactate under normal oxygen conditions. Most of the pyruvate generated by glycolysis in an activated T cell is converted into lactate. As a byproduct of this lactate production, NAD+ is produced which is an electron carrier used in glycolysis and OXPHOS, thus speeding up these processes. Furthermore, glucose-6-phosphate, a glycolysis intermediate, can be shuttled to the pentose phosphate pathway which will aid in nucleic acid and aromatic amino acid synthesis. The pentose phosphate pathway will also produce the reducing agent NADPH which is important in biosynthesis pathways. In addition to glucose metabolism, the activated T cell can also metabolize glutathione through glutaminolysis. Glutamine will be converted into α-ketoglutarate which will be fed into the TCA cycle and further generate ATP (van der Windt and Pearce, 2012).

CD28 engagement activates the Protein Kinase B (AKT)/ mammalian Target of Rapamycin Complex 1 (mTORC1) pathway and induces several changes to initiate anabolic metabolism. This includes the integration of the GLUT1 receptor (a glucose transporter) on the cell surface to induce more glucose intake rather than breakdown intracellular glucose stores. Other transporters activated by this pathway are CD71, for transferrin, and CD98 for amino acids. mTORC1 will also activate Hypoxia Inducible Factor 1α (HIF1α) which upregulates GLUT1 transcription. Additionally, mTORC1 will

activate myelocytomatosis oncogene (MYC) which regulates glutamine uptake (Galgani et al., 2015).

In addition to increased glycolysis, the activation of lipid synthesis enzymes is also necessary. mTORC1 will relieve inhibition of fatty acid synthesis (FAS) through the enzyme acetyl-CoA carboxylase 1 (ACC1; Galgani et al., 2015). mTORC1 promotes fatty acid synthesis by blocking fatty acid oxidation (FAO) by inhibiting the rate-limiting enzyme carnitine palmitoyltransferase 1 (CPT1a) and activating sterol regulatory element-binding (SREBP) proteins. Fatty acid synthesis is favorable for biomass accumulation as fatty acids are crucial to lipid raft synthesis, myristoylation, palmitoylation, and other functions necessary for T cell activation and proliferation (Lochner et al., 2015). All together, these pathways yield more energy and biomass accumulation to support the many new processes initiated by the activated T cell.

1.2.3 Metabolic processes of regulatory T cells

Tregs differ in their metabolic requirements as compared to helper T cells. The energy demands of Tregs are heavily reliant on TCA to fuel OXPHOS, rather than on glycolysis. Tregs mainly use fatty acid oxidation (FAO) to generate acetyl-coA to fuel the TCA cycle. Long chain fatty acids are attached to carnitine through CPT1a. Carnitine shuttles the fatty acids to the mitochondria where it will be converted into acyl-coA *via* carnitine palmitoyltransferase 2. The fatty acids will be further broken down and fed into the TCA cycle, promoting energy production via TCA, and resulting in ATP production through OXPHOS. This leads to increased reactive oxygen species (ROS) and increased mitochondrial mass (Kempkes et al., 2019).

Glycolysis can be very inhibitory to Treg suppression. Tregs have less GLUT1 on their cell surface than helper T cells and the presence of GLUT1 correlates with reduced Treg suppressive capacity. Furthermore, induction of glycolysis in Tregs decreases levels of Foxp3 in these cells (Shi and Chi, 2019). Reciprocally, Foxp3 can downregulate glycolysis by suppressing MYC to further enhance OXPHOS. Tregs rely heavily on OXPHOS, as inhibiting Complex I in the electron transport chain led to less suppressive Tregs (Angelin et al., 2017). However, Tregs also require some level of glycolysis for proliferation and migration. Therefore, Tregs must maintain a balance between glycolysis and FAO that will allow them to maintain suppressive capacity while also accommodating their need for growth (Kempkes et al., 2019).

1.2.4 Metabolism and plasticity

After stimulation by TCR and CD28 signaling, the newly activated T cell will receive cytokine signals from APC to achieve full activation. Differential cytokine signaling induces the T cell to upregulate a specific differentiation program. This differentiation process was originally perceived to be terminal. However, it has been shown that T cells exhibit plasticity in the context of highly inflammatory environments both *in vitro* and *in vivo* (Gerriets et al., 2015; Zhou et al., 2009; Almeida et al., 2016; Galgani et al., 2015). In the highly inflammatory environments of autoimmune diseases, such as in colitis and rheumatoid arthritis, populations of Tregs have been identified that express the Th17 master transcription factor, RORγt. Additionally, these RORγt-expressing Tregs can produce and secrete the signature Th17 cell cytokine, IL-17 (Ren and Li, 2017). Furthermore, both *in vitro* and *in vivo* studies showed that Th17 cells can transdifferentiate into Foxp3-expressing T cells, in response to certain conditions, and this reprogramming

improved disease survival in mouse-models of Th17-mediated autoimmunity. Populations of cells have been identified that co-express Foxp3 and RORγt and that secrete IL-17, but to a lower extent, compared to RORγt-positive T cells. (Almeida et al., 2016; Galgani et al., 2015; Gerriets et al., 2015; Ren and Li, 2017; Zhou et al., 2009).

TGF-β is a key regulator of the observed plasticity that exists between Tregs and Th17 cells. In response to TGF-β signaling, CD4 T cells upregulate both Foxp3 and RORγt. The protein domain encoded by exon 2 of Foxp3 allows for its binding to RORγT. However, when TGF-β is accompanied by signaling initiated by the proinflammatory cytokine IL-6, Foxp3 is downregulated, allowing the induction of the Th17 program (Ren and Li, 2017; Zhou et al., 2009).

Tregs have been shown to take on Th cell phenotypes in response to cytokine stimulation. These signals will tune the Treg to begin expressing the Th cell signature transcription factors and specific chemokine receptors. Some of these Tregs lose Foxp3 expression and become “exTregs”, capable of taking on pathogenic phenotypes (Raffin et al., 2019; Scheinecker et al., 2019). There is also data suggesting Treg cells that take on characteristics of Th cells are necessary for efficient suppression. For example, upregulating T-bet, the Th1 signature transcription factor, has been shown to be beneficial for Treg suppression of Th1 cells (Raffin et al., 2019; Scheinecker et al., 2019). Moreover, Tregs that adopt Th cell phenotypes, stably express this phenotype *ex vivo* (Duhen et al., 2012). However, it is unclear whether proinflammatory cytokine signaling causes Tregs to become exTreg or to improve their suppressive capacity. It is thought that IL-2 signaling may play a role; however, there is no conclusive data that support this hypothesis (Raffin et al., 2019; Scheinecker et al., 2019). Furthermore, because nTregs are more

phenotypically stable than iTregs, it has been suggested that iTregs are more susceptible to becoming exTregs (Raffin et al., 2019).

One major difference between Tregs and Th17 cells is their differential use of metabolic pathways. Tregs rely primarily on FAO, whereas Th17 cells are highly glycolytic, and oxidize glutamine, as their primary energy resources (Priyaharshini et al., 2018; Gerriets et al., 2015). Due to these distinct differences in metabolism, metabolic regulators have been targeted to control Treg-Th17 plasticity (Ren and Li, 2017). Numerous *in vitro* and *in vivo* studies have shown that Treg and Th17 cells can transdifferentiate in the presence of pharmacological compounds that modulate metabolism (Ren and Li, 2017; Mucida et al., 2007; Ozay et al., 2018; Gualdoni et al., 2016; Wang et al., 2016; Sun et al., 2017).

1.3 Graft versus Host Disease pathology and treatment modalities

1.3.1 Failure of immunological tolerance induces autoimmunity

Immunological tolerance is defined as the ability of the immune system to differentiate between self and non-self. There are two modes of immunological tolerance: central and peripheral. Central tolerance occurs in the thymus. As discussed, thymocytes are challenged with self-antigens during thymic education. This challenge ensures that these thymocytes would not mature into self-reactive T cells. However, despite this process, there are still some autoreactive T cells that develop. The same can occur in maturing B cells. Although there are mechanisms to delete self-reacting B cells, some will still enter the peripheral lymphoid tissue and secrete autoantibodies. Peripheral tolerance is mediated by the circulating Tregs (Wang et al., 2015).

When tolerance to self-tissue is not maintained, autoimmunity can occur, and result in widespread inflammation and tissue destruction in the absence of pathogens. The breach in tolerance occurs when autoreactive T cell clones slip out of the thymus. B cells can also be autoreactive, and when not regulated properly in the bone marrow can also slip into the periphery (Wang et al., 2015). Additionally, breach in tolerance can occur as a result of low numbers of Tregs, Tregs that are defective, and/or Tregs that are phenotypically unstable (Chavele and Ehrenstein, 2011). Autoimmunity is becoming more common, affecting over 3% of the population and present with over 100 different pathologies (Wang et al., 2015). There are several etiological factors subscribed to the onset of autoimmune conditions, including genetic background, diet, and environmental factors (Sharif et al., 2017). Due to the heterogeneity of these diseases, there has been an effort to find global mechanisms that contribute to autoimmune conditions, such as Treg insufficiencies, to identify therapeutic targets (Wang et al., 2015).

1.3.2 Graft-versus-Host Disease

Allogeneic stem cell transplantation (ASCT) is a widely used treatment that is curative for some hematological disorders and cancers. The graft replenishes the bone marrow with platelet, red and white blood cell progenitors, and rejuvenates thymic and peripheral lymphoid niches. Prior to transplantation, an immunosuppressive conditioning regimen is administered to eliminate the host's immune cells (Moutuou et al., 2018; Nassereddine et al., 2017). Figure 1.7 illustrates the process of ASCT. A major adverse side effect of ASCT is the onset of Graft-versus-Host Disease (GvHD). GvHD occurs when the donor T cells present in the stem cell graft become activated and attack the host tissue. GvHD can develop as an acute condition within 100 days after transplant, or as a

chronic response which occurs after 100 days post-transplant (Nassereddine et al., 2017).

Acute GvHD is characterized by three phases: initiation, induction, and effector phases. The initiation phase results from inflammation that occurs from the conditioning regimen intended to suppress the host immune system. However, the conditioning can lead to tissue destruction which initiates an inflammatory response, a cytokine storm, and leads to the activation of host APCs. As a result, these APCs will begin to present host antigens (self-antigens) to donor and host T cells. The APCs will present foreign MHC proteins with self-tissue, and activation of T cells making them autoreactive (Nassereddine et al., 2017). The induction phase is marked by T cell migration, activation, and expansion, leading to T cell-specific cytokine release. The effector phase results from cytokines activating cytotoxic T cells which, in turn, mediate tissue destruction (Zeiser et al., 2016). Figure 1.8 illustrates the pathology of acute GvHD. This inflammation can be further perpetuated by aberrations in the immune system's intrinsic regulation.

One way in which the immune system facilitates effector cell responses is through Treg suppression. Pro-inflammatory cytokines released during the cytokine storm can inhibit Treg differentiation and suppressive functions, creating favorable conditions for a pro-inflammatory T cell response (Schlöder et al., 2017). Despite prophylactic therapies, approximately 50% of ASCT recipients will develop acute GvHD (Zeiser et al., 2016). ASCT is still considered a frontline therapy to treat hematologic malignancies and other immune disorders due to its curative potential. This curative potential is driven by the Graft-versus-Leukemia (GVL) effect which results from the ability of alloreactive T cells to destroy malignant cells (Moutuou et al., 2018). As such, a major therapeutic goal is to

block the adverse effects of GvHD while maintaining the beneficial GVL effects after ASCT.

1.3.3 Treatment modalities for GvHD

The host needs to be pre-conditioned prior to the graft transplant to ensure proper engraftment of the transplant. These regimens such as radiation cause tissue damage which elicits the cytokine storm that ultimately results in the induction of disease. There have been efforts to deplete T cells from the graft prior to transplantation. However, this diminishes the GVL effect, reduces engraftment potential, and can also increase other immunological implications for the patient such as susceptibility to viral infections. The current first-line treatment for acute GvHD is a prophylactic course of corticosteroids, which acts as a general immunosuppressant. About half of patients with GvHD present as steroid-refractory diminishing the survival rate to 5-30%. In addition, once GvHD becomes steroid-refractory, there are very few non-steroid treatments currently available to manage the tissue destruction. In recent years, mesenchymal stem cell (MSC) therapy treatments have been investigated. There have been successes with this treatment for some patients who present with less severe disease and primarily for those with skin GvHD, (Nassereddine et al., 2017; Hill et al., 2018).

New, more targeted treatments for GvHD are being investigated such as modifications to the MSC therapies and CAR-T cell therapies. CAR-T cell therapies are very intriguing as they specifically target the alloreactive cells without inhibiting the entire immune system. The type of CAR-T cell used for treatment differs between malignancies, as different CAR T-cells target different surface proteins. One example of the use of CAR-

T cells in GvHD is for patients with B cell lymphomas. These patients receive lymphocyte depleted grafts with CAR-T cells engineered *in vitro* from patient cells with a transgenic receptor that recognizes CD19 on the B cells. This allows for the graft to replenish the hematopoietic niche, prevent alloreactivity, and retain an anti-tumor response. However, the difficulty with this approach lies in distinguishing target proteins on the malignant cell's membrane for the CAR-T cells to target (Smith et al., 2018).

Another non-steroidal approach is to modulate the cytokine signaling that drives the GvHD response. Inhibiting JAK/STAT signaling has shown promising results; however, due to the redundancy of JAK/STAT in the immune system there are off-targets effects (Hill et al., 2018). As a result of JAK/STAT signaling, excessive amounts of proinflammatory cytokines are produced in the extracellular milieu. These cytokines act to drive effector T cell function while dampening iTreg function. To subdue GvHD responses, the iTregs need to be able to suppress an inflammatory environment. However, mainly due to IL-6 signaling, iTreg function is often inhibited. A new treatment modality for GvHD has been to use iTreg therapy. iTregs are isolated from cord blood and expanded *ex vivo*. This approach has been effective, however, expanding the iTreg population is difficult. Additionally, it is not known how plastic these cells are *in vivo.* Due to the presence of IL-6 and other proinflammatory cytokines, it is possible that these iTregs can be converted to an effector T cell, as this phenomenon has been observed both *in vitro* and *in vivo*. As such, more research is needed to resolve the mechanisms behind IL-6 signaling and iTreg plasticity (Hill et al., 2018; Yurchenko et al., 2012; Chen et al., 2009; Ramlal and Hildebrandt, 2017; Fisher et al., 2017).

1.3.4 iTreg therapeutic potential in GvHD

Autoimmune diseases are often treated with widespread immunosuppressive compounds such as steroids, alkylating agents, and antimetabolites (Ferreira et al., 2019). However, these drugs are widespread and not specific to the issue. Over the last 10 years, strategies for developing efficacious Treg therapy have been extensively studied. Treg therapy requires the isolation of Treg cells from the peripheral blood, which constitutes only 2-10% of blood cells, and expand them *ex vivo*. Alternatively, naïve CD4 T cells can be isolated from peripheral blood and differentiated *in vitro* and then re-administered to the patient as adoptive cell therapy (Horwitz et al., 2019).

The motivation for creating Treg therapies came from GvHD studies. When the Treg cells in the grafts were removed, the GvHD manifestation was more severe and when Treg cells were added back into the graft, GvHD was controlled (Sharabi et al., 2018). The first human trial of Treg therapy included two individuals who developed GvHD: one chronic and one acute. In both patients CD4+ CD25+ CD127- T cells were isolated from blood and expanded *ex vivo,* (Trzonkowski et al., 2009) in the presence of CD3/CD28 beads and IL-2, (Trzonkowski et al., 2008). In the chronic GvHD case, the number of CD4+ Foxp3+ cells doubled, and the patient was able to reduce steroid therapy. Additionally, the patient's serum proinflammatory cytokines decreased. However, the amount of IL-10 also decreased so it is not entirely clear how efficacious the overall therapy is in combating the disease other than the alleviation of steroid-usage. In the acute GvHD patient, there was only minor improvement and no increase in levels of Foxp3+ cells (Trzonkowski et al., 2009).

Since the piloting study in 2009, there have been over 50 clinical trials for Treg therapies registered in the US alone. Most studies have determined that Treg therapy is safe but ineffective. It has been determined that for effective suppression the Treg infusion should contain 10^6-10^7 Treg cells. This is difficult to achieve *in vitro,* so many groups have tried to use excessive IL-2 stimulation to increase the proliferation of *in vitro*-derived Treg cells. Despite the use of IL-2, the expansion capacity is variable between donors (Ferreira et al., 2019).

In Type I diabetes, there have been successful cell transfers of Tregs that were tracked up to two years after infusion and were found to be a safe treatment. There were increases in the Treg expression levels of CCR7, CD38 and CD45RO all suggesting good Treg function. However, the majority of Tregs did not last long in the patient (Bluestone et al., 2015). In a trial of Tregs to suppress systemic lupus erythematosus associated inflammation, the Treg survival was sustained over the 12-week study. However, instead of suppressing the Th1-mediated inflammation, after the trial the inflammation was skewed towards a Th17-phenotype (Dall'Era et al., 2019). Interestingly, both studies demonstrated, pre-transfusion, the demethylation of the TSDR of the *Foxp3* locus of the Treg cells to be transfused to show the stability of the population. Despite this, the Treg infusion did not show significant suppression and in the Dall'Era trial it may have made the inflammation worse. It would be of interest to isolate Tregs after several weeks of therapy to determine if the TSDR is still demethylated and/or if the cells still have suppressive capacity.

One of the biggest hurdles with Treg therapy is how to stabilize the Treg phenotype *in vivo* after vigorous *ex vivo* expansion. Several trials have determined that the Treg

numbers are consistent after weeks of after the transfusion. However, despite the absolute Treg numbers presented there is not significant suppression, thus these Treg cells may develop defects in the inflammatory autoimmune environment. Several groups have tried to increase Treg stability by blocking expression of inhibitory proteins or ectopic expression of Foxp3 in the *in vitro* Treg culture, (Ferreira et al, 2019). However, it is unclear if these techniques would stabilize the Treg cells in the face of highly inflammatory environments.

1.4 The multifaceted roles of LKB1 in T cells

1.4.1 An overview of LKB1 biology

LKB1 is a serine/threonine kinase that was first discovered as a protein expressed in the developing fetal liver. In 1998, LKB1 mutations were identified as a mechanism for the development of the rare disease Peutz-Jeghers syndrome. This syndrome induces a predisposition to sporadic tumoral growth. Interestingly, the disease-causing mutation is a loss-of-function mutation, indicating the tumor suppressive capacity of the kinase (Hemminki et al., 1998). LKB1 normally resides in the cytoplasm in a complex with a pseudokinase, STRADα, and a scaffolding protein, MO25. Most kinases will be activated by a phosphorylation of a residue in the alpha helix of the activation loop thus exposing the kinase domain. However, LKB1 is activated by an unconventional mechanism. Concisely, STRADα, acting as a pseudokinase, persists in a closed conformation to allow for the interaction of MO25 and LKB1. MO25 will allow the exposure of the active conformation of LKB1 and thus allow LKB1 to bind and phosphorylate substrates (Zeqiraj et al., 2009).

LKB1 is encoded by the *Stk11* gene which is located on chromosome 19p. *Stk11* can be alternatively spliced into three forms: long, short, and ΔN. The long form has 10 exons and a 5' UTR, encoding a 433 amino acid protein whereas the short form has 9 exons, encoding a 404 amino acid protein in humans (Zhu et al., 2013). These are the main isoforms that are found amongst various tissue types. Figure 1.9 illustrates the exon and intron arrangements of these two isoforms. The ΔN form was found as a mutant that has oncogenic properties. This isoform is spliced starting from exon 3, and found predominantly in skeletal muscle and heart tissue (Dahmani et al., 2014; Thibert et al., 2015).

The translated protein has a nuclear localization sequence on the N-terminus, followed by the kinase domain. The C-terminus has modification sites for phosphorylation and farnesylation. The short form has the entire kinase domain but has a different c-terminus thus there is no farnesylation site and the phosphorylation sites differ (Zhu et al., 2013). The ΔN form does not have the canonical N-terminus and thus does not have a nuclear localization signal (Dahmani et al., 2014; Thibert et al., 2015). Interestingly, despite the differences between all these forms, all three forms can take part in canonical LKB1 signaling (Zhu et al., 2013; Dahmani et al., 2014; Thibert et al., 2015). Prior to the activation of LKB1, the nuclear localization signal confines the protein to the nucleus where it cannot function. The protein will be phosphorylated by Protein Kinase Cζ which releases LKB1 from the nucleus to the cytoplasm where it interacts with downstream targets (Zhu et al., 2013). The ΔN form does not have a nuclear localization signal nor catalytic capacity due to an incomplete kinase domain. However, through some unknown mechanism can activate downstream targets. This is postulated to occur through binding-

induced conformational changes on downstream targets (Dahmani et al., 2014; Thibert et al., 2015).

1.4.2 AMPK activation in T cells

The main substrate of LKB1 is 5' adenosine monophosphate kinase (AMPK). AMPK is a heterotrimeric energy sensor that allows the cell to respond to nutrient starvation and revert to catabolic energy pathways for energy production. AMPK has three subunits: α, β, and γ. The α subunit is the kinase domain that can phosphorylate serine and threonine residues and the β and γ subunits bind adenylate nucleotides. When AMP binds to the γ subunit of AMPK, the kinase is protected from dephosphorylation of the Threonine 172 activation site and induces the kinase activity of AMPK. AMPK must be phosphorylated on residue Threonine 172 for full activation. Interestingly, it has been shown that during initial T cell stimulation, the associated calcium flux induces CAMKK activation and CAMKK can activate AMPK. However, the main upstream activator of AMPK is LKB1. Once both AMP bind and AMPK is phosphorylated at Threonine 172, AMPK can phosphorylate downstream substrates to ensure catabolic pathways dominate the energetics of the cell (Ma et al., 2017; Blagih et al., 2012; Lochner et al., 2015).

One way in which this occurs is through the phosphorylation of acetyl coA carboxylase (ACC) which catalyzes the reaction of acetyl-coA to malonyl coA. Malonyl-coA will drive lipid synthesis and inhibit carnitine palmoyl transferase 1 (CPT1) which is essential for lipid oxidation. AMPK will phosphorylate ACC at serine 9 thus deactivating it, leading to the inhibition of lipid synthesis and relieving the inhibition of lipid oxidation. Another way in which AMPK drives catabolic energy is through the phosphorylation of the TSC complex. When TSC1/2 are phosphorylated, it blocks the activation of mammalian

target of rapamycin (mTOR) which engages the cell in processes such as protein synthesis and glycolysis. Figure 1.10 illustrates some of the ways in which the LKB1/AMPK pathway can regulate cellular energetics towards catabolic processes (Ma et al., 2017; Blagih et al., 2012; Lochner et al., 2015).

1.4.3 LKB1 in effector T cells

The role of AMPK in mature peripheral T cells, has been studied extensively (Ma et al., 2017). However, several studies over the past decade have demonstrated that there are AMPK-independent functions of LKB1 in T cells. One of these functions is that LKB1 regulates the survival and proliferation of T cells. Upon activation, T cells will undergo rapid proliferation; however, if LKB1 is conditionally ablated, this proliferation is obstructed, irrespective of the addition of CD28 (MacIver et al., 2011; Tamás et al., 2010). Additionally, T cells lacking LKB1 have survival deficiencies, presumably from inadequacy of the energy demands put forth by rapid proliferation. This is presumably because these cells expressed higher amounts of Bax, a proapoptotic BCL-2 family protein, then wild type cells upon metabolic stress. However, this survival defect can be circumvented by transgenic expression of BcL-xL (MacIver et al., 2011).

Unsurprisingly, LKB1 also has an impact on T cell metabolism. $LKB1^{-/-}$ T cells have increased glycolytic rates and elevated expression of glycolytic enzymes. Additionally, in the absence of LKB1, the uptake of glucose is increased due to increased expression of the glucose transporter, GLUT1. Regarding effector function, $LKB1^{-/-}$ T cells show increased activation as evidenced by CD44 expression and greater cytokine production. When $LKB1^{-/-}$ CD4 T cells were polarized *in vitro* towards a Th1, Th2, or Th17 phenotype, all subsets showed increased cytokine production as compared to WT. This

phenomenon seems to be unique to LKB1, as AMPK$\alpha^{-/-}$ CD4 T cells did not show increased cytokine production. CD8 T cells that were activated in the absence of LKB1, also showed increased cytokine production of IL-17A and IFNγ; however this effect was mirrored in AMPK deficient CD8 T cells. Interestingly, the increased levels of IFNγ in both the LKB1-/- and AMPK$\alpha^{-/-}$ CD8 cells was diminished upon addition of rapamycin, suggesting that the increased IFNγ expression is due to the increased mTORC1 induced by deficiency of either LKB1 or AMPK (MacIver et al., 2011).

1.4.4 LKB1 in regulatory T cells

LKB1 has been extensively studied as a metabolic regulator in times of cellular stress. Treg cells exhibit a metabolic phenotype that is analogous to cellular starvation with the increased usage of autophagy and fatty acid oxidation, as compared to effector T cells. As such, over the past several years the role of LKB1 in Treg cells has become of interest. Several studies have shown that ablation of LKB1 in Tregs induces widespread autoimmunity and interestingly, the effect on Treg cells are AMPK-independent. It has been suggested that the effect of LKB1 on Treg cells may be mediated by other kinases downstream of LKB1 such as MAP/mitochondrial affinity-regulating kinases (MARKs) and salt-inducible kinases (SIK; He et al., 2017).

The first study to evaluate the contribution of LKB1 in the Treg phenotype examined the relationship between Foxp3 and LKB1. This study demonstrated that LKB1 is highly expressed in Treg cells as compared to conventionally stimulated CD4 T cells. Furthermore, this study presented evidence that LKB1 stabilizes Foxp3 expression by blocking STAT4 which methylates CNS2 on the *Foxp3* locus (Wu et al., 2017). The

specific role of LKB1 in the inhibition of CNS2 methylation was also shown in human Treg cells from patients with acute GvHD. These patients had lower frequencies of Treg cells and the Treg cells were highly unstable as the CNS2 region was demethylated compared to healthy controls (Su et al., 2019). LKB1 has been shown to contribute to TGF-β signaling as LKB1 floxed Tregs have reduced TGF-β signaling due to the lower expression of TGF-β receptors (Wu et al., 2017).

LKB1 also reduces co-receptors OX-40, PD-1, and GITR expression on Treg cells to inhibit type 2 inflammation. This modification occurs through modulation of β-catenin activity thus leading to widespread inflammation (Yang et al., 2017). Interestingly, LKB1-deficiency in dendritic cells allows for increased Treg proliferation. This occurs through increased expression of OX-40 ligand on the dendritic cells in the absence of LKB1 leading to increased proliferation which is attributed to more contact between the Treg and dendritic cells. Further, the authors demonstrated that LPS or bacterial stimulation of dendritic cells led to the complete abrogation of LKB1 in dendritic cells. This suggests that LKB1 may act as a rheostat of Treg activity during infection (Chen et al., 2018).

As expected, LKB1 deficiency reduces the rates of fatty acid oxidation in Treg cells. Furthermore, LKB1-deficient Treg cells show overall perturbation in the mitochondria as evidenced by lower mitochondrial mass, less reactive oxygen species, lower mitochondrial membrane potential, and defects in oxidative phosphorylation. All of this leads to the reduced quantity of intracellular ATP in the cells. This is presumably one of the reasons for the low survival rates of Treg cells in the absence of LKB1 (He et al., 2017; Yang et al., 2017). Additionally, LKB1-deficient Treg cells demonstrate reduced activity of the mevalonate pathway which lead to reduced expression of Foxp3 and

subsequently less suppressive capacity. Interestingly, LKB1-deficency and subsequent aberration of the mevalonate pathway led to the Tregs expressing IFNγ and IL-17A, suggesting phenotypic instability (Timilshina et al., 2019).

1.5 PKCθ in T cell signaling

1.5.1 PKCθ in T cell activation

Protein kinase Cθ (PKCθ) is a T-cell specific kinase that functions in T cell activation and induction of effector function. PKCθ is serine/threonine kinase from the Ca^{++}- independent Protein Kinase C family, which is activated by diacylglycerol (Isakov and Altman, 2002). T cell signaling induces the hydrolysis of phospholipase C to diacylglycerol, which binds to the C1 domain of PKCθ and tethers the protein to the immunological synapse created between the T cell and APC. Recruitment of PKCθ to the immunological synapse allows for the interaction between PKCθ and LCK and LCK acts as a bridge PKCθ and CD28 (Brezar et al., 2015).

PKCθ regulates the activity of NF-kB upon T cell activation which culminates in IL-2 production. NF-κB transcription factors are sequestered in the cytoplasm by IκB phosphorylation. T cells have the CARMA1-BCL10-MALT1 (CBM) complex that has the capacity to ignite the signaling pathway to release NF-kB. After PKCθ is recruited to the immunological synapse it will phosphorylate CARMA1 which will allow for the formation of the CBM complex. MALT1 will activate the IκB complex (IKK) and lead to the degradation of IκB and the release of NF-κB transcription factors from the cytoplasm (Isakov and Altman, 2002; Lucas et al., 2004; Brezar et al., 2015).

PKCθ also has a direct role in gene expression in T cells. Sutcliffe et al., 2011 showed for the first time that PKCθ is catalytically active in the nucleus as well as in the cytoplasm in activated T cells. PKCθ binds to active chromatin and forms a transcriptional complex with RNA polymerase II, histone kinase mitogen and stress activated kinase-1 (MSK-1), 14-3-3ζ, and lysine specific demethylase 1 (LSD1). This complex binds to promoters of T cell activation genes such as *cd69* and microRNAs to modulate T cell function (Sutcliffe et al., 2011; Sutcliffe and Rao, 2011).

1.5.2 PKCθ has opposing functions in effector and regulatory T cells

PKCθ is crucial for Th17 cell differentiation as PKCθ$^{-/-}$ T cells exhibit a diminished Th17 phenotype. There are several Th17 differentiation mechanisms that require PKCθ function. IL-6 signaling is necessary for Th17 differentiation. Downstream of IL-6, STAT3 is activated and induces the upregulation of RORγt and IL-17. PKCθ has been shown to regulate *Stat3* expression, (Kwon et al., 2013). Furthermore, PKCθ activates sterol regulatory complex 1 (SRC1) which regulates the transcription of *Rorc,* the transcript for RORγt. As such, PKCθ is crucial for Th17 cell differentiation and effector function, through modulation of IL-17 production (Sen et al., 2018).

In Tregs, PKCθ has been shown to have an inhibitory role. PKCθ$^{-/-}$ mice exhibit increased Treg number *in vivo*, and an increased capacity for *in vitro* Treg differentiation. PKCθ signaling modulates the activity of the AKT pathway. In the absence of PKCθ, AKT phosphorylates transcription factors Foxo1/3a which drives Foxp3 upregulation (Ma et al., 2012). PKCθ also affects the suppressive capacity of Treg cells. PKCθ is sequestered away from the immunological synapse in Treg cells to prevent Tumor necrosis factor-

alpha (TNF-α) production. This has been shown to prevent the Treg from undergoing TNF-α mediated inactivation of the Treg. Furthermore, in autoimmune models, if PKCθ is inhibited on the Treg cells, the Treg cells are more efficacious in suppression in the pro-inflammatory autoimmune environment (Zanin-Zhorov et al., 2010).

1.6 RNA processing

1.6.1 RNA splicing

Upon transcription by RNA polymerase II, immature messenger RNA (mRNA) transcripts will undergo a variety of modifications during RNA maturation. Maturation of the RNA allows for its export from the nucleus to the cytoplasm to the ribosome for translation. Three major modifications occur during the maturation process: 5' capping, 3' polyadenylation, and splicing (Bentley, 2014). The 5' cap and 3' polyadenylation protect the transcript, allow for nuclear export, and aid in the translation process. The mature RNA will also require non-coding introns to be spliced out of the transcript to allow for the coding exons to be ligated together. In addition to the basic splicing out of introns, alternative splicing can occur. Normally, constitutive splicing will ligate exons in the linear sequence in which they appear. However, alternative splicing confers great diversity of potential gene products by allowing for exons in different regions of the transcript to be ligated together. Even more diversity can be introduced by changing the established intron boundaries in the transcript. These measures allow for the possibility of drastically different versions, or isoforms, of the same transcript (Black et al., 2019).

RNA splicing is a highly regulated and complex process that requires the formation of the spliceosome, a multiprotein complex that recognizes splicing sites and induces

splicing of the transcript. The spliceosome contains five small nuclear ribonucleoproteins (snRNPs) U1, U2, U4, U5, and U6, which work in unison to accurately identify splice sites on the transcript. U1 is the first component to bind and binds at the 5' splice site. The U2 auxiliary factor (U2AF) heterodimer will then recognize and bind to the 3' splice site (Black et al., 2019). The heterodimer is made up of the U2AF35 and U2A65 subunits which bind, respectively, to pyrimidine stretches and the AG dinucleotide found at the junction of the intron and exon (Shenasa and Hertel, 2019). The U2AF heterodimer recruits and aids in the incorporation of the U2 unit. The U2 unit will bind by base pairing with the branch point site. Subsequently the U4.U6/U5 tri-snRNPs are recruited into the splice site. The proteins in this complex will act to drive a transesterification reaction to ligate the exons and release the intron. Figure 1.11 depicts the overall splicing reaction (Black et al., 2019).

1.6.2 RNA binding proteins

Alternative splicing occurs in over 90% of the human genome conferring the great diversity seen within the human proteome. Interestingly, the different isoforms generated from a single gene tend to be expressed in a tissue-specific fashion and are regulated by the splicing code. Within the splicing code are *cis* and *trans* acting elements that modulate the strength of the splice site and the resulting splice variant (Neil and Fairbrother, 2019).

Alternative splice sites are usually weak but can be significantly enhanced by *trans*-acting elements, RNA binding proteins (RBPs). To enhance splicing, the RBP binds to the *cis*-element, the intron splicing enhancer (ISE), or the exon splicing enhancer (ESE) which is located within the material to be spliced out. The RBP acts as a flag, promoting splicesome complex formation on the site and stabilizing the interaction. Conversely, RBP binding to the intron splicing silencer (ISS) or exon splicing silencer (ESS), acts to inhibit

splicesome binding to the splice site (Black et al., 2019). A schematic of this process is found in Figure 1.12. There are two main families of RBPs: serine and arginine-rich (SR) proteins and heterogenous nuclear ribonucleoproteins (hnRNP). SR proteins tend to associate more with enhancers while hnRNP proteins associate more frequently with silencers. However, it has been demonstrated that both proteins can bind enhancers or silencers, both, making the splicing code much more nuanced than originally thought (House and Lynch 2008).

1.6.3 HnRNP proteins in T cell activation and function

There are 20 major members in the hnRNP protein family. These proteins can affect transcription by modulating transcript expression, stability, and transport. The hnRNP proteins have a nuclear localization signal and reside primarily in the nucleus. However, some hnRNPs also have a cytoplasmic function regulated by post-translational modifications or recruitment by other proteins which allow translocation of the hnRNP from the nucleus to the cytoplasm. There are four RNA-binding motifs common to the hnRNP family: the KH domain, the RNA recognition motif (RRM), the quasi-RRM, and the arginine-glycine-glycine (RGG) motif. Although there is redundancy in the RNA binding sequences, diversity is conferred by additional auxiliary domains and post-translation domains (Geuens et al, 2016).

There are many genes that are alternatively spliced in T cells. These include genes encoding cytokines, cytokine receptors, kinases, transmembrane receptors, and intracellular signaling proteins. Differences in stimuli, cell phenotype, or even malignant transformation can all drive differential splicing of these genes. The canonical example of

hnRNP-mediated splicing effects on T cell activation is through the splicing of CD45 (Lynch, 2004).

CD45 is a transmembrane protein tyrosine phosphatase that is encoded by the gene *Prprc* (Holmes, 2006). CD45 function is crucial to T cell activation because it dephosphorylates the inhibitory Y505 phosphorylation of LCK, allowing for the subsequent activation signaling cascades to ensue (Zamoyska, 2007). CD45 is alternatively spliced into 5 different protein-coding variants that result from splicing of exons 4-6 and produce isoforms with different extracellular domains. Different isoforms are expressed in different cell phenotypes and correspond with the cells' maturity and activation status. In naïve peripheral T cells, the CD45RA isoform is expressed. As the cell becomes activated it will produce the CD45RO isoform, which lacks exons 4-6. Additionally, memory T cells are also characterized by the expression of CD45RO. CD45RO is considerably smaller than CD45RA and, as such, allows the CD45 proteins to dimerize more easily. Once dimerized, the phosphatase ability is sterically inhibited, acting as a feedback loop to curtail excessive activation of the cell (Lynch 2004).

CD45 splicing is regulated by hnRNPL and hnRNPLL. However, hnRNPLL is upregulated by CD28 engagement and is thought to more directly control T cell activation-mediated CD45 splicing (Butte et al., 2012; Oberdoerffer et al., 2008). To generate CD45RO, hnRNPLL binds to exons 4 and 6 at the respective ESS. When hnRNPLL is bound to both exons, it forms a stable complex due to cross-exon interaction and allows for exon 5 to be included in the lariat that is spliced out of the transcript. HnRNPL can also bind exon 4 but cannot bind exon 5 or 6 thus hnRNPL, alone, is unable to generate the CD45RO isoform (Preuβner et al., 2012; Figure 1.13).

In addition to regulating CD45, hnRNPLL has been shown to be a global splicing regulator in activated T cells. HnRNPLL expression and the number of spliced transcripts, both, are increased following CD28 engagement. There are several other genes that are targets of hnRNPLL that have been identified that could affect T cell differentiation and function, such as CD44, STAT5a, and fatty acid synthase (Butte et al., 2012).

1.7 Rationale and hypothesis

Naïve T cells circulate through peripheral lymphoid tissues until they encounter antigen in the context of peptide:MHC presented by an APC. The T cell will receive two signals from surface receptors on the APC, and a third signal from cytokines in the microenvironment, to fully engage the T cell activation program. Initially, IL-2 signaling through autocrine and paracrine networks, is necessary for full T cell activation and proliferation. Additional cytokine signals will induce the cell to differentiate into specific effector states. APCs secrete different cytokines, depending on the nature of the engulfed antigen, to prime the T cell to specifically target this type of pathogen. The cytokine signal will drive upregulation of a signature transcription factor in the T cell, which will further induce acquisition of effector function, mainly through effector cell cytokine production (Zhou et al., 2009).

It was originally thought that T cell differentiation is an irreversible phenomenon, but several studies have demonstrated that T cells can reacquire the signature identity of a different effector phenotype, through a process known as plasticity. Plasticity can occur between several different T cell subsets but is observed most markedly between iTreg and Th17 cells (Zhou et al., 2009). TGF-β signaling is crucial in the differentiation of both subsets through its upregulation of Foxp3 and RORγt, both. Foxp3 associates

with the DNA binding domain of RORγt, to render RORγt incapable of modulating gene expression, thus allowing Foxp3 to drive a suppressive program (Zhou et al., 2008). In the presence of TGF-β, together with the pro-inflammatory cytokine IL-6, Foxp3 becomes acetylated which alters its ability to bind to target genes, and thus allows RORγt to translocate to the nucleus and activate the Th17 differentiation program (Samanta et al., 2008).

In some autoimmune diseases, such as in psoriasis and rheumatoid arthritis, iTregs can take on Th17 functions, such as IL-17 production, *in vivo*, especially in the context of a highly inflammatory environment. However, it is not clear whether these Tregs retain their suppressive capacity and this ability may, in fact, be disease specific. For example, in colitis models, populations of FOXP3+ RORγT+ Tregs were identified that retained their suppressive capacity, whereas Th17-producing Tregs enhanced the inflammatory pathology seen in psoriasis. *In vitro*, iTregs can be reprogrammed to adopt a Th17 phenotype in the presence of Th17-inducing cytokines, such as IL-6 (Ren and Li, 2017). However, the question remains how these signals are integrated and how they affect immunological tolerance.

An emerging regulator of the balance between effector T cells and regulatory T cells is PKCθ. Treg cells receive negative feedback from TCR/CD28 signaling and resultant PKCθ and AKT activation. However, Treg cells also need to build an immunological synapse with an APC for proper activation (Zanin-Zhorov et al., 2010). In effector T cells, TCR/CD28 signaling induces PKCθ to translocate within lipid rafts to form the central region of the immunological synapse (IS) which is formed upon TCR:APC interaction. IS recruitment brings PKCθ into close proximity with its substrate, CARMA1,

and ultimately leads to the liberation and translocation of NF-κB transcription factors to the nucleus where they mediate gene expression (Brezar et al., 2015). However, in Treg cells, PKCθ is not recruited to the immunological synapse, thus protecting Tregs from TCR-mediated inactivation and from TNF-α-mediated inhibition of suppressive activity. Inhibiting PKCθ activity also preserves Foxp3 expression (Zanin-Zhorov et al., 2010).

In contrast, PKCθ is essential for Th17 cell differentiation and effector function. PKCθ-deficient Th17 cells express less RORγt and secrete less IL-17. PKCθ has several effects on Th17 phenotype acquisition. PKCθ, in coordination with NF-κB and AP-1, stimulates the expression of STAT3, an adaptor molecule which transmits signals through the IL-6 receptor (Kwon et al., 2012). Additionally, PKCθ phosphorylates steroid receptor coactivator (SRC) which, when phosphorylated, mediates RORγt-DNA binding and drives IL-17 production. Phosphorylated SRC also drives Foxp3 degradation, relieving Foxp3-mediated inhibition of RORγt (Sen et al., 2017).

How changes in the metabolic profiles may affect the plasticity of different cell subsets represents another area of active inquiry. Naïve CD4 T cells are metabolically quiescent and rely on oxidative phosphorylation as their primary means of generating cellular energy (Chapman et al., 2019). Following activation, effector T cells undergo metabolic changes to accommodate the increased energy demands that accompany proliferation effector functions. TCR and CD28 engagement upregulate glycolytic pathways, including the mTORC1 pathway, to facilitate increased glucose transport. In contrast, Treg cells utilize fatty acid oxidation and oxidative phosphorylation to derive energy and can thrive in environments where glucose availability is low (Blagih et al., 2012; Gerriets et al., 2015). It is curious that there exists such a high level of plasticity

between two cell types that utilize markedly different metabolic profiles. In recent years, our group and others have shown that T helper cell phenotype can be changed by pharmacologically manipulating cellular metabolism. However, a clear mechanism describing how these metabolic switches occur, and whether they drive, or result from, changes in Treg-Th17 cell plasticity has yet to be fully elucidated.

Liver Kinase B1 (LKB1) is a metabolic regulator that drives energy processes like FAO. As Tregs preferentially metabolize fatty acids, the role of LKB1 in Treg biology has become of interest. Over the past several years, different studies have been shown that LKB1 protects Treg function by maintaining metabolism, cellular survival, and Foxp3 expression (He et al., 2017; Timilshina et al., 2019; Wu et al., 2017; Yang et al., 2017). In acute GvHD patients, their Tregs had very low levels of LKB1 which correlated with low Foxp3 expression, suggesting a role of LKB1 in the maintenance of peripheral tolerance (Su et al., 2019). In addition, when LKB1 was deleted from Tregs *in vivo*, these cells started expressing pro-inflammatory cytokines (Timilshina et al., 2019). ~~This suggests that LKB1 regulates phenotypic stability. However, is this through the effect on Foxp3 or does LKB1 have an intrinsic effect on stability?~~

These pieces of data suggest that LKB1 is crucial to Treg identity in regard to Foxp3 expression and resultant suppressive capacity. However, is this a result of Foxp3 expression itself or does LKB1 have an intrinsic effect on phenotypic stability. In this study, our central hypothesis is that LKB1 regulates the plasticity axis between Th17 cells and iTregs.

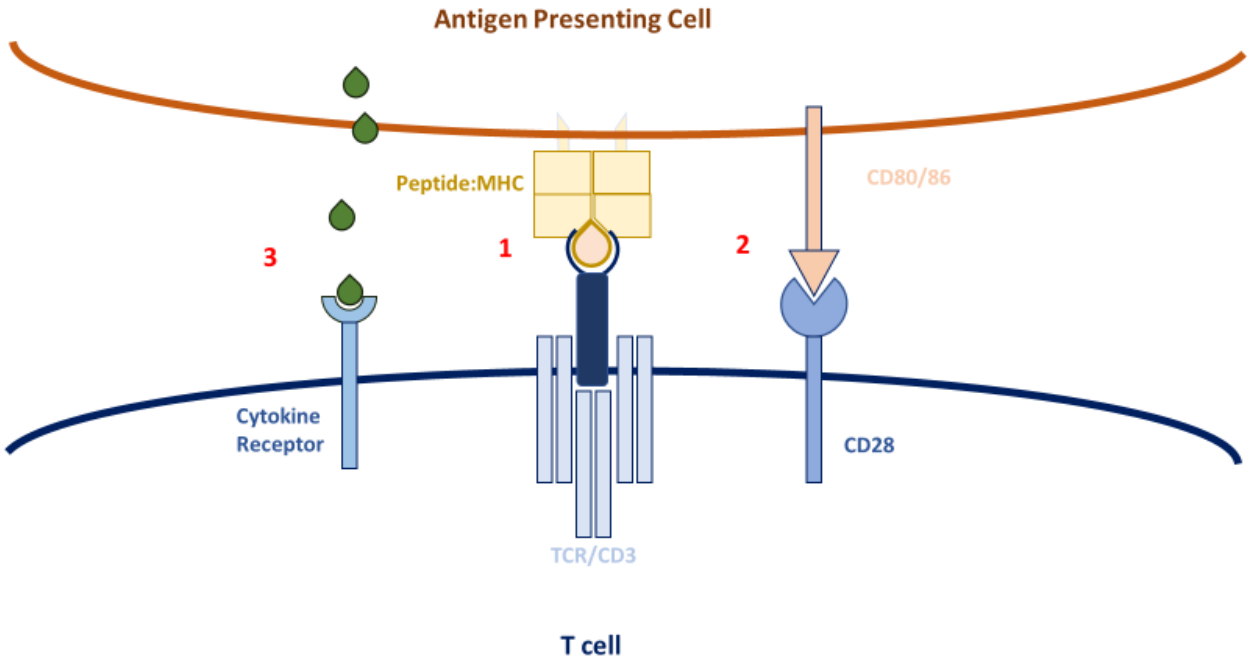

Figure 1.1 T cell activation

T cells require three signals for complete activation and the induction of proliferative and survival pathways. Signal 1 is mediated through the TCR and MHC association. Signal 2 is mediated through CD28 and CD80/86. Signal 3 is mediated through cytokine signals.

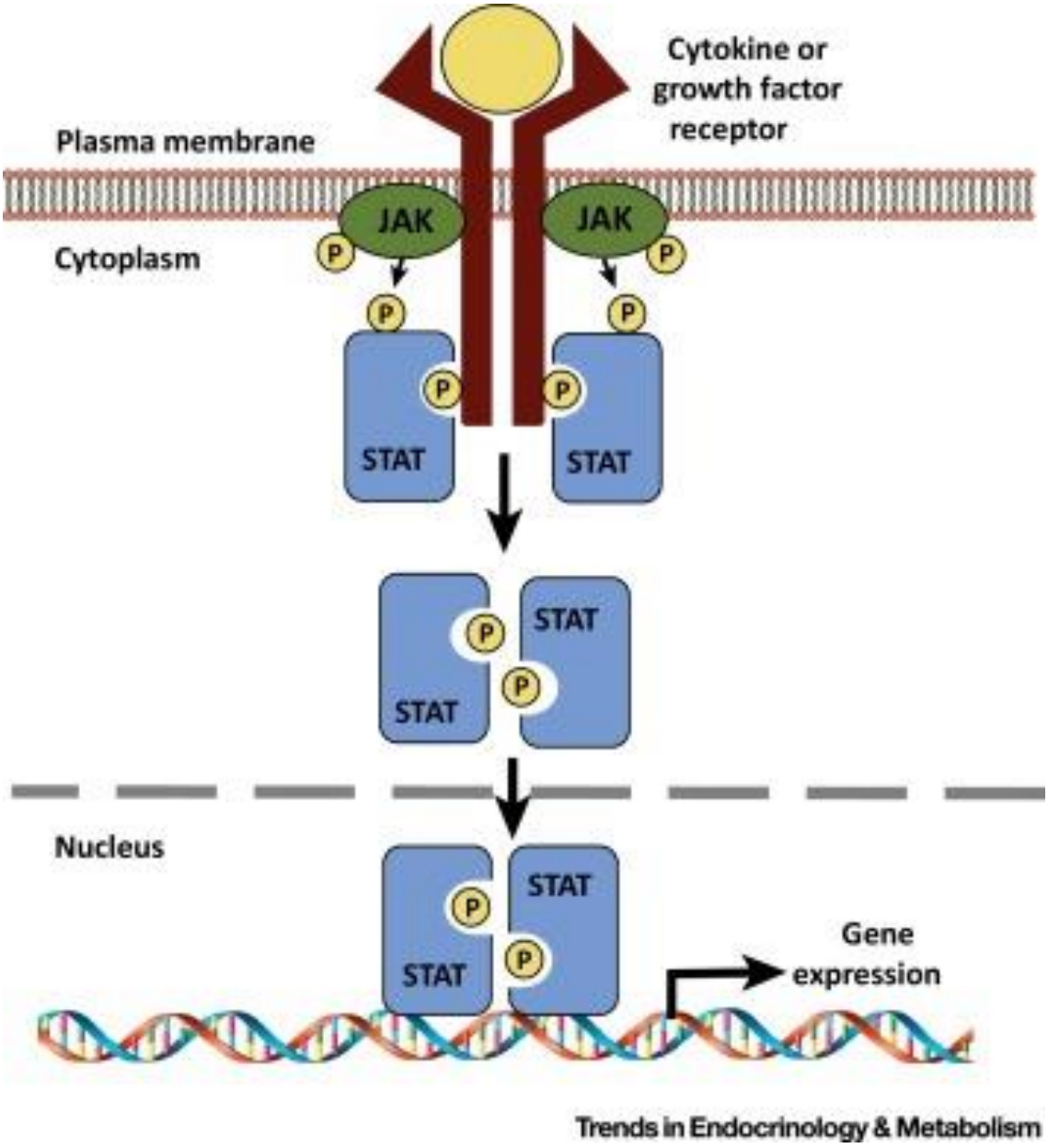

Figure 1.2 JAK/STAT signaling

Cytokines bind a specific receptor to illicit a cellular response. JAK kinases are bound to the cytokine receptor and become autophosphorylated when ligand binds the receptor. Activated JAK phosphorylates STAT protein, exposing a Sh2 domain which allows STAT proteins to dimerize. STAT dimers can enter the nucleus and modulate gene expression. Adapted from Dodington et al., 2018.

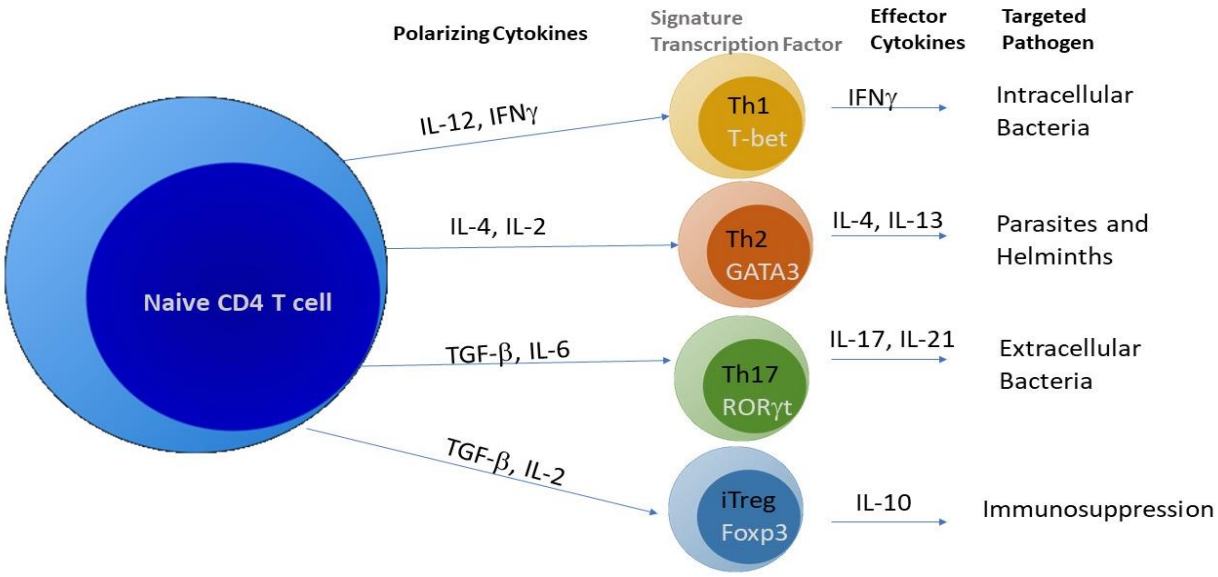

Figure 1.3 CD4 helper T cell subsets

A naïve CD4 T cell will receive cytokine signaling from the APC that has activated it. The specific cytokine signals will upregulate the signature transcription factor of the effector subset. The signature transcription factor will induce the production and secretion of cytokines that are most efficacious at targeting the specific pathogen.

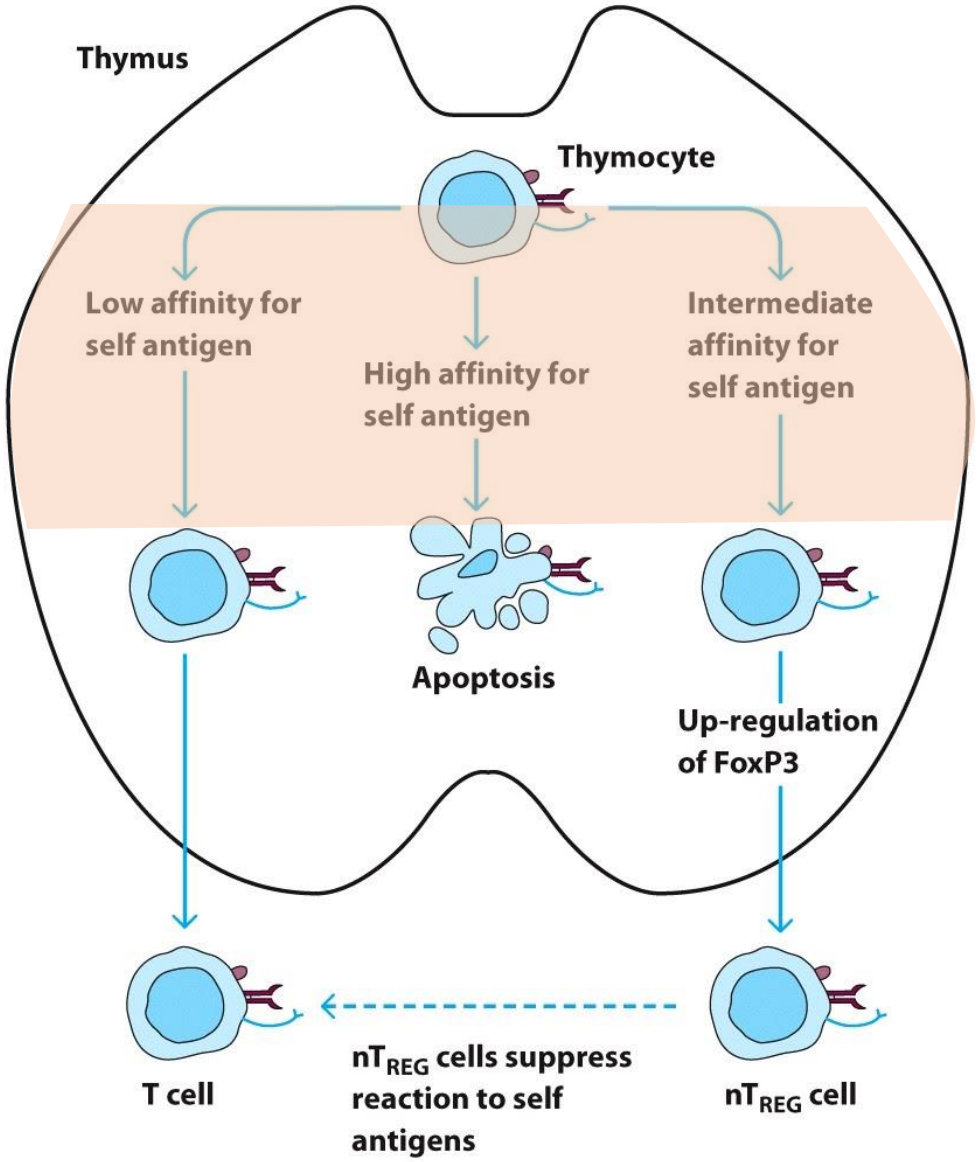

Figure 16-2
Kuby Immunology, Seventh Edition
© 2013 W. H. Freeman and Company

Figure 1.4 nTreg differentiation

Single-positive CD4 thymocytes are challenged with self-antigens produced by *Aire* expression, in orange. If the thymocyte reacts strongly to the self-antigen it will undergo apoptosis. Whereas if the thymocyte reacts moderately to the self-antigen, it will upregulate CD25 and Foxp3, and exit to the periphery until it is activated. Modified from Kuby Immunology, 7th edition

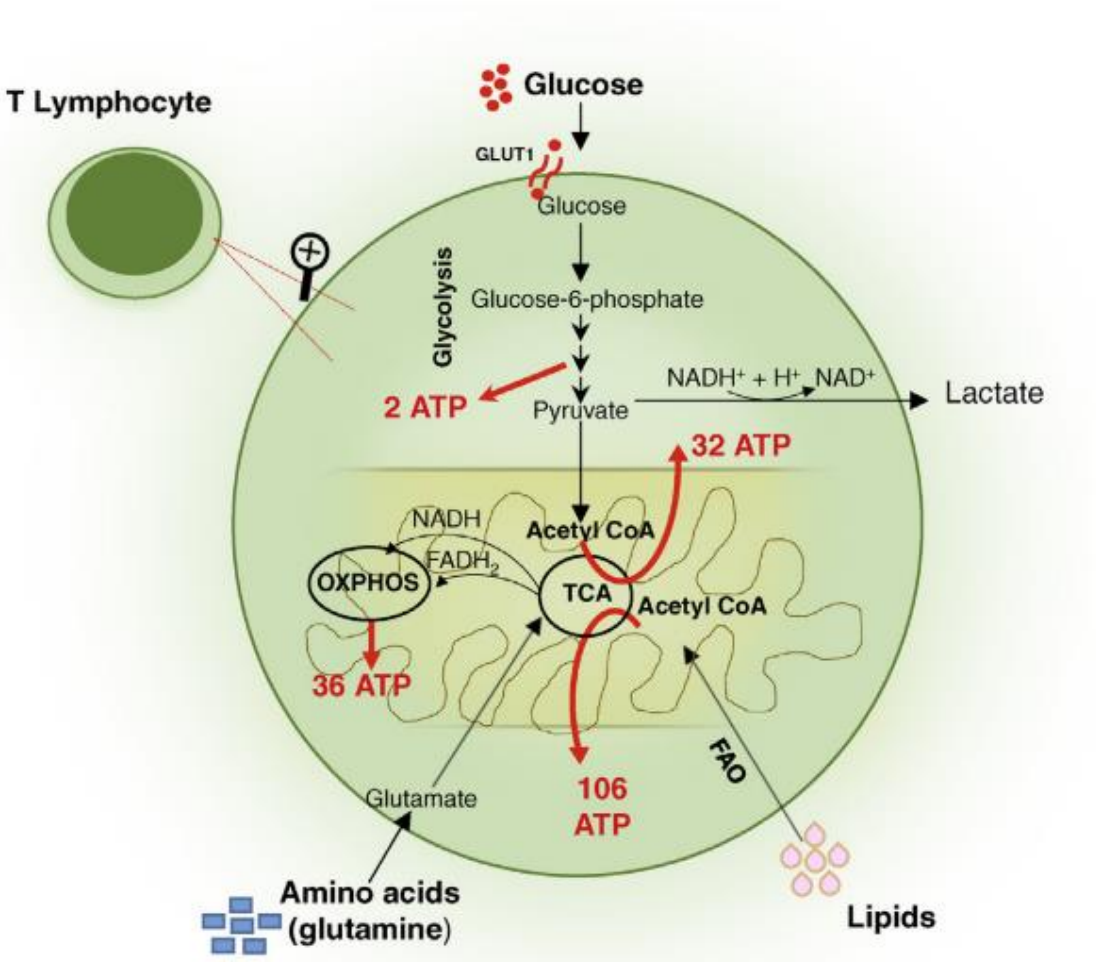

Figure 1.5 Cellular energy production

Different metabolic pathways can be used to generate acetyl-coA and TCA intermediates to fuel the TCA cycle. The TCA cycle will reduce electron carriers to be used in OXPHOS and result in ATP production. Adapted from Galgani et al., 2015.

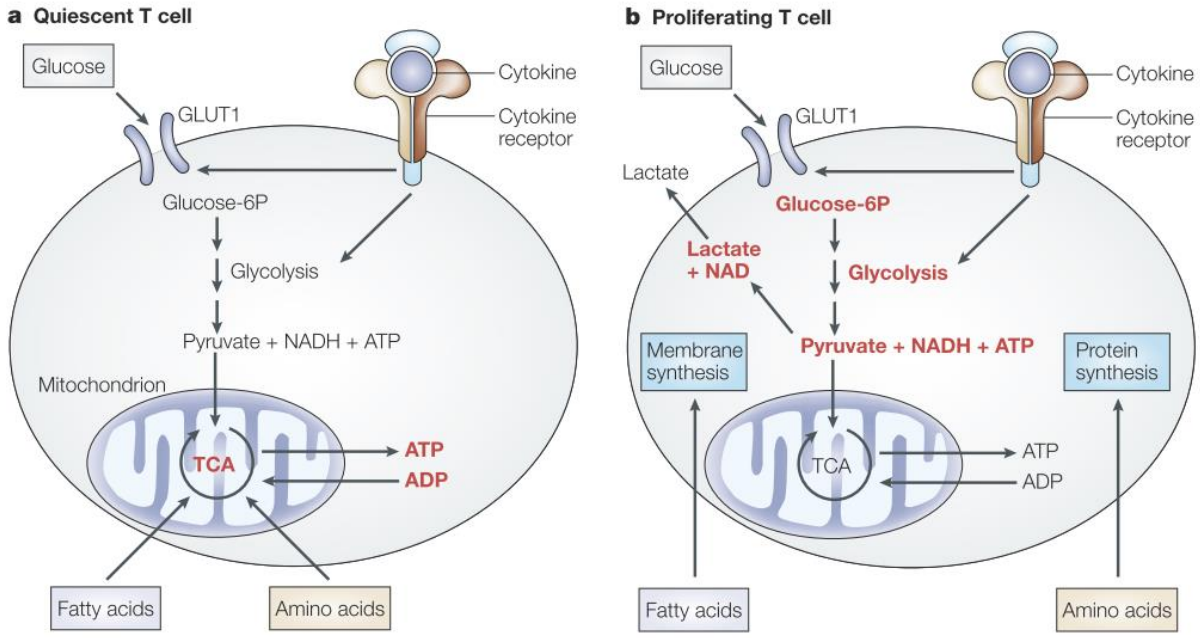

Figure 1.6 The Warburg effect

Upon activation, T cells will undergo the Warburg Effect which induces the cell to undergo glycolysis and convert glucose into lactate under normal oxygen conditions. The lactate production produces NAD+ which will be used for OXPHOS to produce ATP. Adapted from Fox et al., 2005.

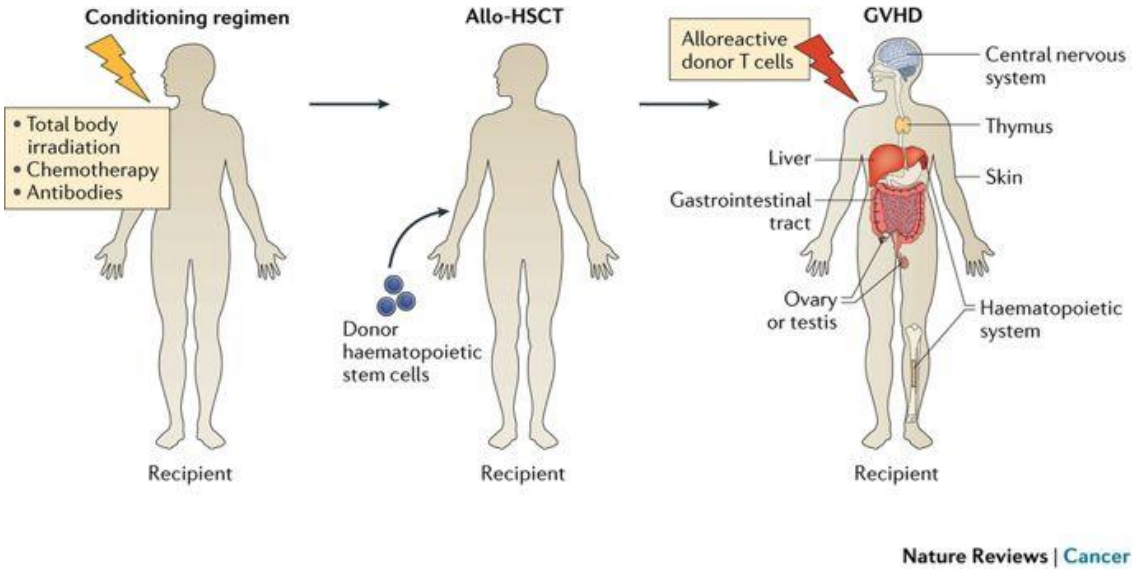

Figure 1.7 ASCT and the onset of GvHD

ASCT is a curative treatment for hematological disorders and cancers. The recipient undergoes a conditioning regimen to deplete the recipient's immune cells. The recipient will then receive a transfusion of the donor cells. The donor cells will kill any remaining cancer cells. However, the donor cells can also attack the recipient's tissue, the manifestation of GvHD. Adapted from Shono and van den Brink, 2018.

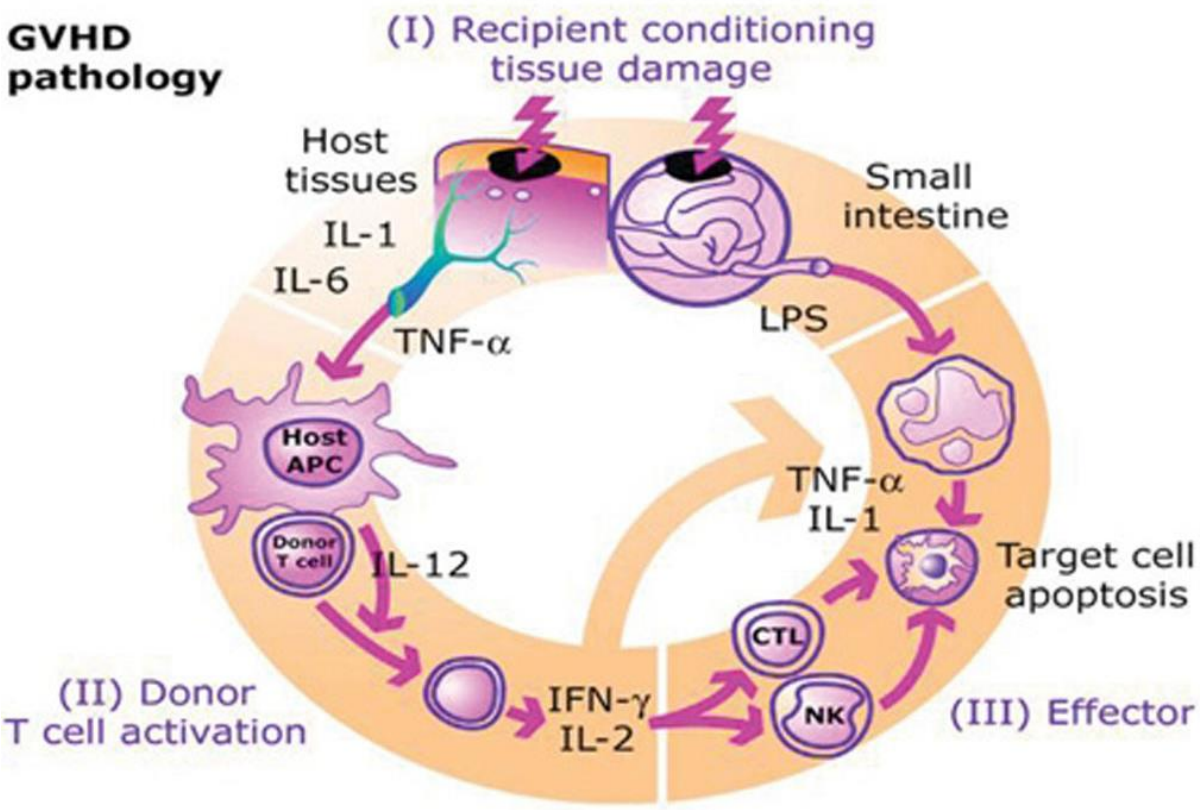

Figure 1.8 The pathology of GvHD

(1) Initiation of GvHD is caused by the pre-conditioning regimen. (2) The conditioning regimen induces a cytokine storm and activates cells in the graft. (3) The activated donor cells will cause tissue damage by reacting to self-antigens and killing recipient cells. Adapted from Ferrara and Levine, 2008.

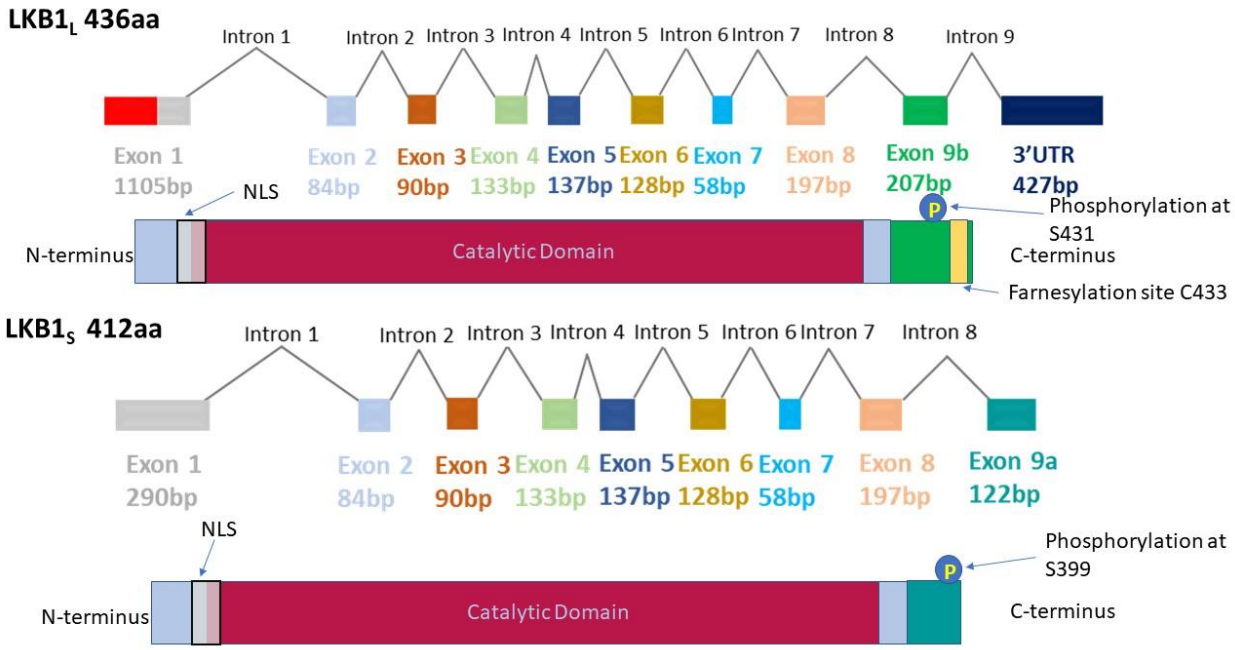

Figure 1.9 The main isoforms of *Stk11*

Stk11 is the gene that encodes for LKB1. There are two main isoforms, long and short. The short isoform is encoded by exon 9a and has a stop codon at the end of the exon, changing the 3'UTR of the transcript. The short form has the entire kinase domain but loses the farnesylation site and has a different phosphorylation site.

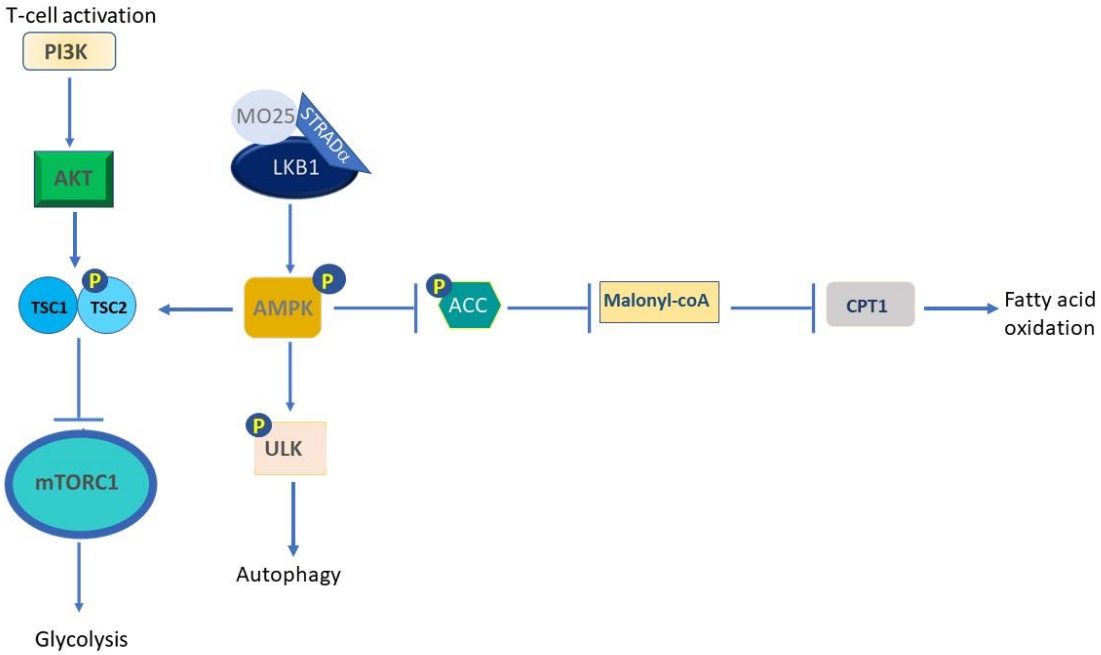

Figure 1.10 The LKB1/AMPK pathway regulates cellular energetics

LKB1 phosphorylates and activates AMPK. AMPK can phosphorylate numerous substrates, all working towards driving the cell towards catabolic energy processes. A few of the substrates of AMPK are illustrated in this figure.

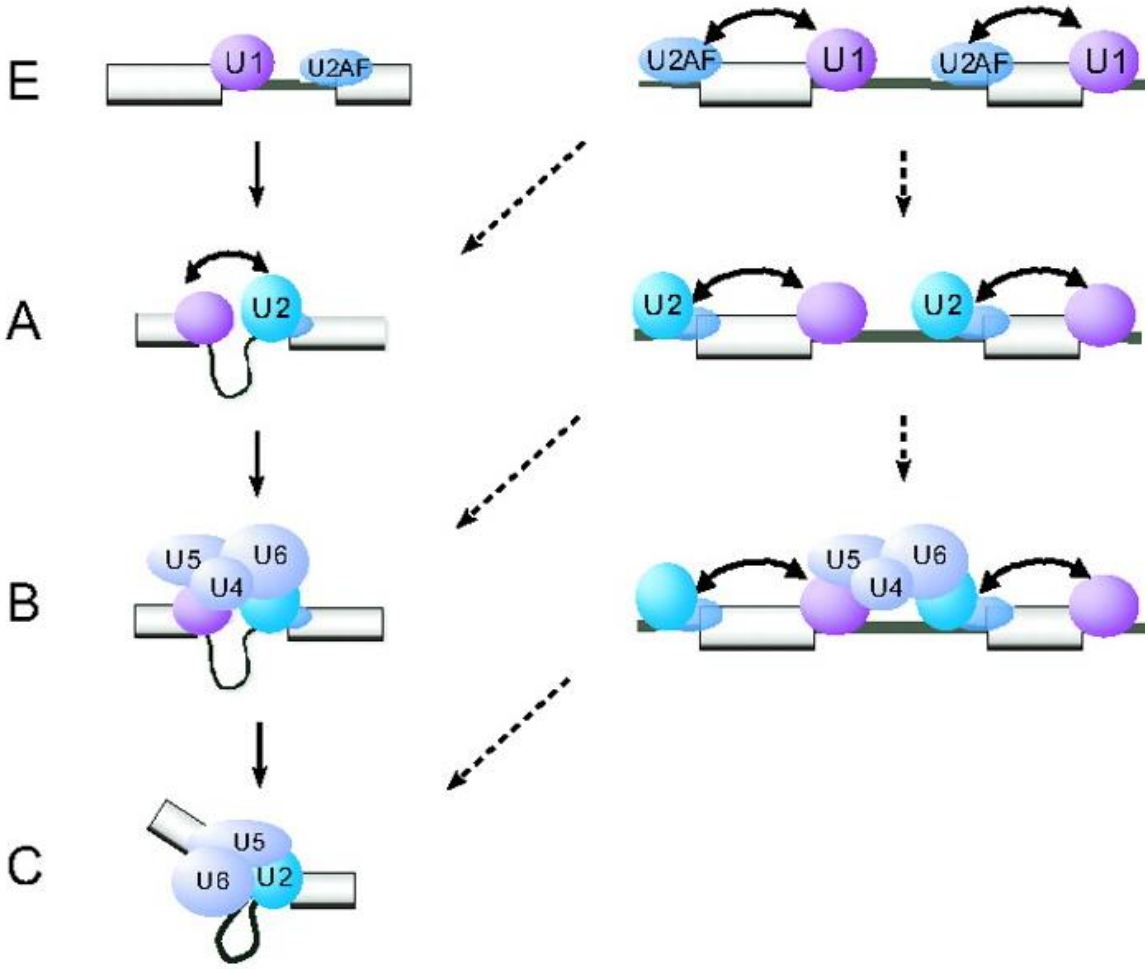

Figure 1.11 Formation of the spliceosome

The spliceosome is a multiprotein complex that recognizes and binds to the splice sites on a transcript. U1 binds to the 5' splice site first. This allows for U2AF to bind to the 3' splice site and recruit U2 to base pair at the branch point site. Once U2 is bound, the U4.U6/U5 tri-snRNPs can bind, induce the transesterification reaction which will release the introns and ligate the exons. Adapted from House and Lynch, 2007.

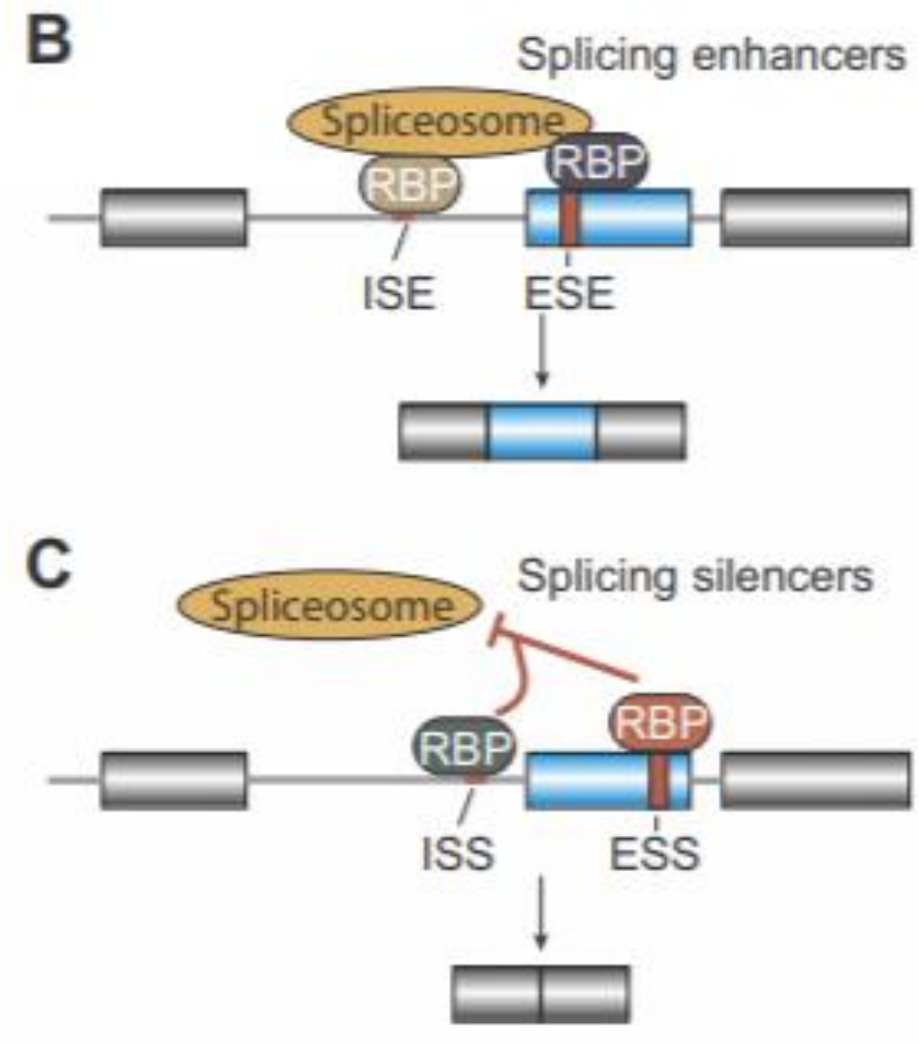

Figure 1.12 RBPs regulate spliceosome formation

RBPs can orchestrate the splicing of a transcript by binding enhancer regions and recruit the spliceosome to bind to the transcript. Conversely, RBPs can block spliceosome recruitment by binding silencer regions and block the spliceosome from binding to the transcript. Adapted from Black et al., 2019.

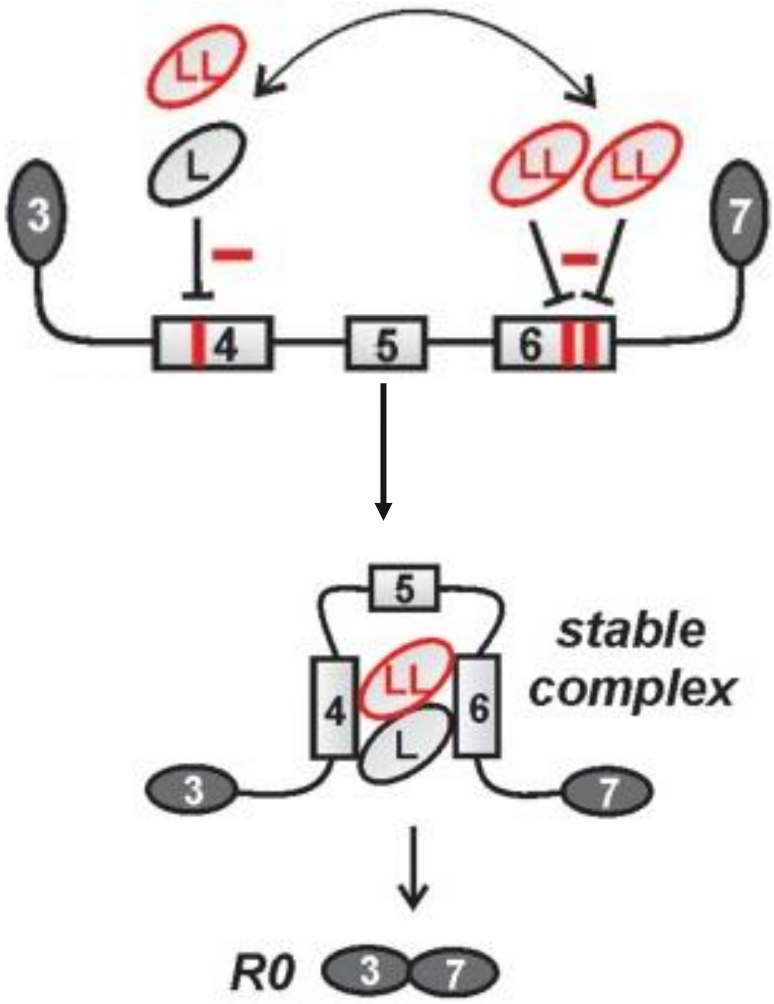

Figure 1.13 HnRNPLL and hnRNPL regulate CD45 splicing

HnRNPLL can bind to exons 4 and 6 to induce the splicing of exons 4-6. However, hnRNPL can only bind to exon 4. Thus, hnRNPL alone cannot generate the isoform CD45RO. Modified from Preuβner et al., 2012.

CHAPTER 2

LKB1 SPLICING IS MEDIATED THROUGH HNRNPLL AND PKCθ Signaling

2.1 Introduction

LKB1 is an essential energy regulator of Treg metabolism (He et al. 2017). As such, we hypothesized that iTreg cells, which mainly utilize fatty acid oxidation for energy, would express more LKB1 compared to Th17 cells, which are highly glycolytic. LKB1 phosphorylates and activates AMPK to perpetuate FAO by subsequently activating CPT1a, a fatty acid oxidation enzyme, and blocking ACC, a FAS enzyme, refer to Figure 1.10 (Lochner et al., 2015; Ma et al., 2017). LKB1 has numerous functions in Treg cells, with the most important being maintaining Foxp3 expression. Stable Foxp3 expression has been shown to be LKB1-dependent through its inhibitory effects on CNS methylation. Furthermore, LKB1 ablation correlates with loss of Foxp3 expression. Whether this is achieved by maintaining open chromatin, through increased protein stability, or both, has yet to be established (He et al., 2017; Wu et al., 2017; Yang et al., 2017). However, to our knowledge, no one has yet explored how LKB1 may contribute to iTreg-Th17 plasticity. To this end, we hypothesized that LKB1 would not be expressed in Th17 cells and that expression of LKB1 would maintain the Treg phenotype.

2.2 Results

2.2.1 LKB1 is expressed in both Th17 and iTreg cells

To determine if LKB1 expression differs between iTreg and Th17 cells, we needed to develop a protocol that would provide a homogenous and stable cell

population. Primary CD4+ T cells were negatively selected prior to differentiation *in vitro*. We used RORγt+ expression to define Th17 cells and CD25+ Foxp3+ expression to identify iTregs, Figure 2.1. We did not assess CD4 expression, as these cells were negatively selected for CD4 expression prior to stimulation and differentiation. To verify that this Treg population was indeed functional, we assessed the presence of nuclear Foxp3, Figure 2.1. We determined that the optimal culturing of naive splenic CD4 T cells to differentiate was over a period of 7 days.

To assess LKB1 expression within the two subsets, we first gated cells on RORγt or Foxp3 expression within Th17 or iTreg cells, respectively, and then measured LKB1 levels *via* flow cytometry. As a control, we stimulated naïve CD4 T cells in the presence of IL-2. These cells are considered stimulated but non-polarized (NP). We found that greater than 90% of Th17 cells and iTregs, both, expressed LKB1. Surprisingly, Th17 cells expressed approximately three times more LKB1 than did iTregs or NP cells, as determined by median fluorescence intensity (MFI), a readout of relative protein expression, Figure 2.2.

LKB1 is activated in the cytoplasm through its association with STRADα and MO25 proteins (Zeqiraj et al., 2009). Prior to its activation, LKB1 resides in the nucleus until phosphorylated by PKCζ, which frees LKB1 from its nuclear constraints (Zhu et al., 2013). We questioned whether, although the Th17 cells expressed more LKB1 than iTregs, was it perhaps retained in the nucleus and thus, not activated? Using AMNIS imaging flow cytometry, we asked whether Th17 cells had more nuclear LKB1 than iTreg cells. Using the IDEAS software and the nuclear similarity algorithm, we determined that there was not a significant difference in the nuclear residency of LKB1

between subsets. We made this determination by analyzing the percent of cells in the population staining positive for nuclear LKB1 together with the nuclear similarity score, Figure 2.2. Although we did not fully confirm the activation status of the protein, it has been demonstrated by others that activated LKB1 resides in the cytoplasm. Since we saw no differences in nuclear LKB1, we presumed that the amount of activated LKB1 may be similar between Th17 and iTreg cells.

2.2.2 LKB1 Isoforms are differentially expressed in Th17 and iTreg cells

LKB1 can exist as one of 2 splice variants: a short ($LKB1_S$) or a long ($LKB1_L$) isoform. LKB1 is encoded by the gene, S*tk11*, which is comprised of 9 exons and a 3' UTR. There are two versions of exon 9, 9a and 9b, with exon 9a only expressed in the short isoform of LKB1. Exon 9a contains a stop codon, leading to a truncated transcript ($LKB1_S$) with no 3'UTR. Exon 9a is a cryptic exon that is spliced from intron 8, Figure 2.3. Given that LKB1 can exist in different forms, we revised our hypothesis to ask whether Th17 cells and iTregs express different isoforms of LKB1. We designed primers to specifically amplify exon 9a in $Stk11_S$, exon 9b in $Stk11_L$, as well as to exon 1 which is expressed in both $Stk11_S$ and $Stk11_L$, and which we refer to as the "common" exon, Figure 2.3. Using qRT-PCR, we determined that Th17 cells express approximately 2-fold more $Stk11_S$ than iTregs, Figure 2.4. The ratio of $Stk11_S$:$Stk11_L$ was significantly higher in Th17 cells, compared to iTregs, indicating that $Stk11_S$ is the predominant form of LKB1 in Th17 cells, Figure 2.4. We also confirmed, by immunoblot, that $LKB1_S$ is more abundantly expressed in Th17 cells than in iTregs, Figure 2.4. Altogether, our data show that Th17 cells express higher levels of LKB1 than do iTregs. Furthermore, Th17

cells and iTregs express different splice variants of *Stk11*, with Th17 cells expressing significantly more *Stk11*$_S$/LKB1$_S$ than iTregs.

2.2.3 HnRNPLL associates with the short *Stk11* isoform

RNA binding proteins (RBPs) help guide components of the spliceosome to newly synthesized transcripts to mediate splicing events (Lynch, 2004). RBPmap is a tool that predicts binding sites on transcripts by RNA binding proteins by identifying binding motifs within transcript sequences (Paz et al., 2014). We analyzed the *Stk11* sequence using RBPmap scan, and identified heterogenous nuclear ribonucleotide protein L-like (hnRNPLL) as one of the RBPs that showed strong binding potential. HnRNPLL seemed like a good target to explore since it has already been shown to have a role in CD45 splicing in T cells (Wu et al., 2008). Therefore, we asked whether hnRNPLL might also have a function in *Stk11* splicing. We measured the expression of hnRNPLL in Th17 cells and iTregs. We found that Th17 cells express more hnRNPLL than iTregs, Figure 2.5. We next asked whether differences in hnRNPLL expression has functional consequences for *Stk11* splicing in Th17 and iTreg cells. We generated whole cell lysates from Th17 and iTreg cells, in which RBPs were bound to RNA through a cross-linking step. We then immunoprecipitated hnRNPLL from the cell lysates to enrich for RNA transcripts that were bound to hnRNPLL. We used the *Stk11*$_S$ primer set to determine whether *Stk11*$_S$ was preferentially associated with hnRNPLL in Th17 cells as compared to iTreg cells. As shown in Figure 2.5, we observed that, in Th17 cells there was significantly more *Stk11*$_S$ transcript bound to hnRNPLL, than in iTregs.

We previously showed we can restrict the function of a target protein using a specific antibody complexed to a synthetic peptide containing a protein transduction domain mimic (PTDM) which will transport it across the cell membrane (Ozay et al., 2016). HnRNPLL was shown to mediate alternative splicing of CD45 in activated in T cells (Wu et al., 2008); therefore, we assessed CD45 splicing as a readout of effective delivery of anti-hnRNPLL. Consistent with previous results, when we delivered an antibody to hnRNPLL into CD4 T cells, we found that anti-hnRNPLL treatment altered the expression of CD45 isoforms (Figure 2.6). To further explore how hnRNPLL mediates *Stk11* alternative splicing, we delivered anti-hnRNPLL into CD4 T cells, then assessed *Stk11* transcript expression after Th17 polarization. Interestingly, we found a large increase in the level of the *Stk11* common transcript, Figure 2.6, perhaps as a compensatory mechanism. However, when we measured the level *Stk11*$_S$ relative to that of the common transcript, we found that anti-hnRNPLL delivery significantly reduced *Stk11*$_S$ expression and the ratio of *Stk11*$_S$:*Stk11*$_L$ in Th17 cells, Figure 2.6. These data confirm that functional hnRNPLL is required for efficient *Stk11* alternative splicing in Th17 cells, and by using a cell penetrating peptide to deliver anti-hnRNPLL in CD4 T cells prior to Th17 differentiation we can modulate the alternative splicing process that generates *Stk11*$_S$.

2.2.4 PKCθ regulates hnRNPLL expression and *Stk11*$_S$ splicing in Th17 cells

PKCθ functions downstream of CD28 signaling and has been shown to be necessary for Th17 differentiation, while also acting to inhibit iTreg function (Ma et al., 2012; Sen et al., 2018; Kwon et al., 2012). Additionally, PKCθ has been shown to regulate the splicing activity of RBPs, such as that of SC-35 (McCuaig et al., 2015).

Interestingly, hnRNPLL protein expression is also upregulated following CD28 engagement (Butte et al., 2012). Therefore, we asked whether PKCθ regulates hnRNPLL-mediated $LKB1_S$ expression in Th17 cells. Compared to iTregs, there was significantly more *Prkcq*, the transcript that encodes PKCθ, in Th17 cells, Figure 2.7. Furthermore, when naive CD4 T cells from $PKC\theta^{-/-}$ mice are stimulated and cultured under Th17 polarizing conditions, they show defective Th17 differentiation, as characterized by diminished *Il17f* production, the Th17 master effector cytokine, Figure 2.7. In Th17 cells that lack PKCθ, *Hnrnpll* transcript expression is abrogated, whereas in Th17 cells that express WT PKCθ, we observed robust levels of *Hnrnpll*, confirming PKCθ is necessary for *Hnrnpll* expression in Th17 cells, Figure 2.7.

We demonstrated that hnRNPLL functions to regulate *Stk11* alternative splicing, Figure 2.6. Therefore, we hypothesized that, in $PKC\theta^{-/-}$ Th17 cells, we would observe defective *Stk11* splicing. We found that, compared to WT Th17 cells, there is significantly less $Stk11_S$ in Th17 cells lacking PKCθ, Figure 2.7. These data suggest that PKCθ regulates hnRNPLL expression in Th17 cells, which further functions to mediate *Stk11* alternative splicing.

2.2.5 Inhibiting PKCθ alters hnRNPLL and LKB1 expression in iTregs

We determined that PKCθ is important for inducing hnRNPLL expression in Th17 cells. Therefore, we hypothesized that deleting PKCθ would reduce the expression of hnRNPLL in iTregs. To test this, we differentiated WT and $PKC\theta^{-/-}$ CD4 T cells into iTreg cells. We evaluated hnRNPLL expression *via* immunoblot and observed a slight reduction in the amount of protein expressed, Figure 2.8. This led us to further investigate the expression of $LKB1_S$ expressed in $PKC\theta^{-/-}$ iTregs. We observed a

reduction in LKB1$_S$, in iTregs that lack PKCθ, Figure 2.8. Although these data did not meet statistical significance, it demonstrates PKCθ does regulate LKB1 alternative splicing, but that there are other signals involved in this pathway.

2.3 Discussion

LKB1 is emerging as an important regulator of Treg function and survival through mechanisms that act through STATs, β-catenin, and microtubule-affinity regulating kinase signaling (He et al., 2017; Wu et al., 2017; Yang et al., 2017). These are in addition to AMPK-dependent LKB1 activities which facilitate fatty acid oxidation and autophagic processes in Tregs. However, whether or if LKB1 functions in Th17 cell differentiation has not been explored previously. We identified a significant increase in LKB1 expression in Th17 cells polarized *in vitro*, compared to iTregs. Further, we presume that the activation of LKB1 is similar between both subsets as we observed that there was no significant difference in the number of cells that express nuclear LKB1 between both subsets. Intriguingly, we determined that *Stk11*, which encodes LKB1, can be alternatively spliced to generate a short splice variant, *Stk11*$_S$, which is expressed abundantly in Th17 cells.

As we sought to further define the regulatory mechanisms responsible for the alternative splicing we observed, we identified through RBPmap that the RBP, hnRNPLL, has strong binding potential on the *Stk11* transcript. We found that the level of expression of hnRNPLL in Th17 cells is much higher than in iTregs. Further leading us to consider that hnRNPLL mediates *Stk11* splicing in Th17 cells. We used RNA immunoprecipitation to confirm that hnRNPLL, could bind to *Stk11*$_S$. We confirmed that functional hnRNPLL is necessary for robust *Stk11*$_S$ processing in Th17 cells by

demonstrating a significant decrease in *Stk11*$_S$ when we inhibit hnRNPLL using a cell-penetrating antibody. We also demonstrated that *Stk11* splicing is a marked feature of a Th17 phenotype. When *Stk11* splicing was inhibited in Th17 cells, through perturbation of hnRNPLL function, there was a concomitant decrease in *Rorc* and increase in *Foxp3* expression.

The T cell specific kinase, PKCθ, appears to function at the nexus of Treg-Th17 cell fate choice, preventing induction of iTreg programming while promoting Th17 differentiation. One means by which PKCθ inhibits Treg function is by mediating TNF inactivation (Zanin-Zhorov et al., 2010). Conversely, PKCθ is required for Th17 differentiation in vitro, serving to stabilize RORγt (Sen et al., 2018). PKCθ acts in various capacities in mature CD4 T cells, from regulating RBPs, such as SC-35, to facilitating NF-κB activation (McCuaig et al. 2015; Shin et al., 2014).

In this study, we demonstrate a relationship between PKCθ and hnRNPLL expression. In PKCθ$^{-/-}$ Th17 cells, *Hnrnpll* was nearly completely abrogated, further implicating PKCθ signaling in regulating alternative splicing machinery. It is unclear how PKCθ affects hnRNPLL expression. Our data are consistent with a model whereby hnRNPLL expression requires intact PKCθ signaling and is reduced in cells that lack PKCθ, Figure 2.9. Interestingly, hnRNPLL expression and function is increased with CD28 co-stimulation (Butte et al., 2012), which has been shown to induce PKCθ phosphorylation and recruitment to the immunological synapse (Zanin-Zhorov et al., 2010). However, is the relationship between hnRNPLL expression and PKCθ a byproduct of CD28 signaling? To test this, CD28 engagement would have to be abrogated. However, this would be a difficult experiment to perform, as T cells

stimulated in the absence of CD28 undergo anergy (Berg-Brown et al., 2004). This will need to be investigated by identifying possible PKCθ functions on hnRNPLL. One intriguing possibility is that PKCθ can directly affect the transcription of *Hnrnpll*. PKCθ has been shown to interact with 14-3-3ζ and NF-κB to form a transcriptional complex at the promoter of various genes to drive cytokine production, including IFNγ and IL-17 (Sutcliffe et al., 2012). This could be tested using a chromatin immunoprecipitation assay by immunoprecipitating PKCθ and using qRT-PCR for *Hnrnpll*. HnRNPLL regulates the splicing of numerous transcripts important in T cells (Butte et al., 2012). As such, further elucidation of the relationship between PKCθ and hnRNPLL will be beneficial in determining the regulation of these transcripts and how this affects T cell function.

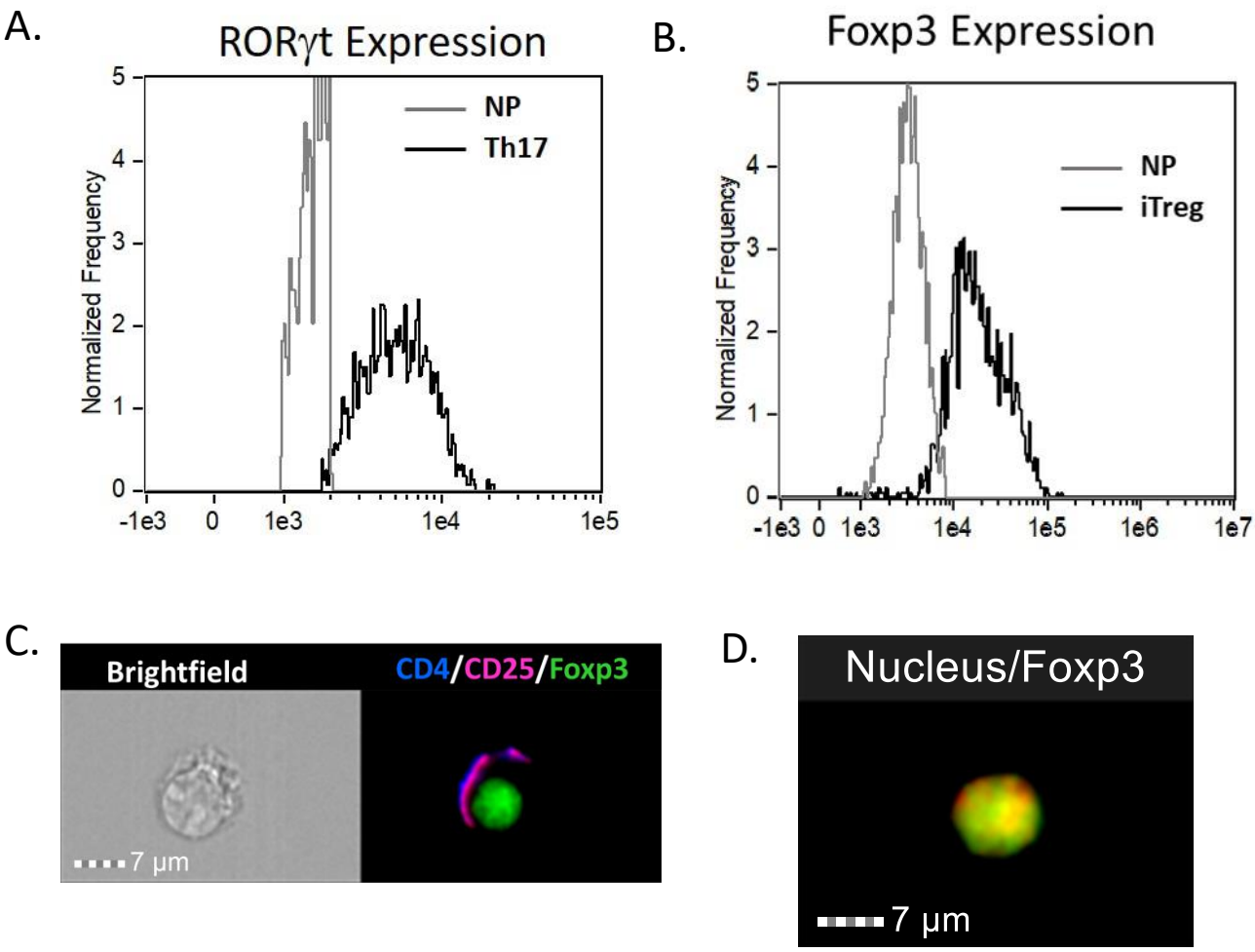

Figure 2.1 *In vitro* polarization of iTreg and Th17 cells

(A) NP and Th17 cells were analyzed for RORγt expression and (B) NP and iTregs were analyzed for Foxp3 expression through flow cytometry. To ensure that the iTregs had an iTreg phenotype, we examined CD25 (C) and nuclear Foxp3 (D) expression using AMNIS imaging flow cytometry.

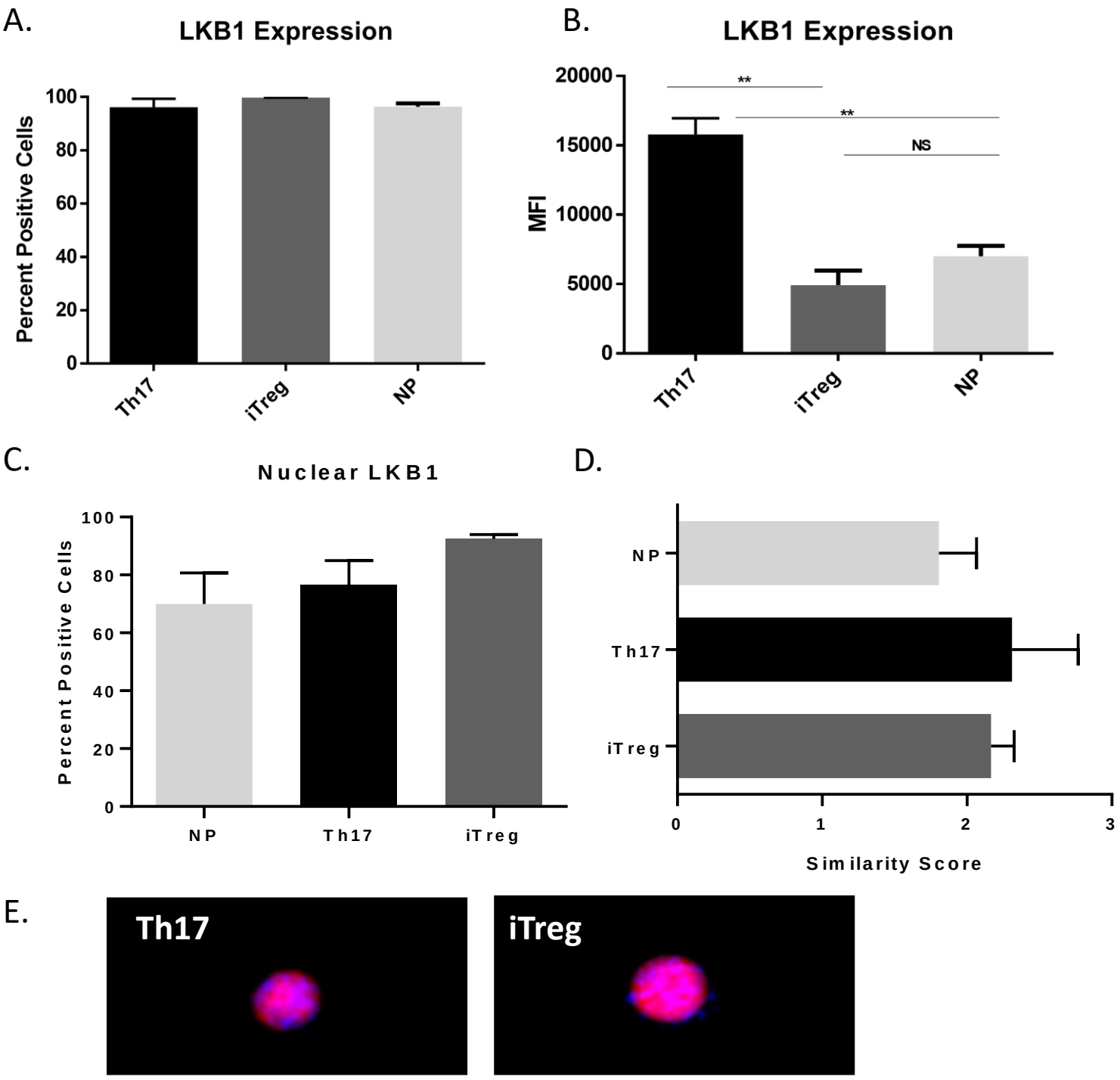

Figure 2.2 LKB1 expression in Th17 and iTreg cells

Naïve CD4 T cells were activated and polarized toward a Th17 or iTreg phenotype. The percent of cells expressing LKB1 (A) and the relative protein expression (B) were measured by flow cytometry. The number of cells expressing nuclear LKB1 (C) and the similarity score (D) was analyzed by AMNIS Imaging flow cytometry. (E) Representative images of nuclear LKB1: the nuclear stain is red, LKB1 is blue, and the overlay is purple. Data are the mean ± S.E.M of three independent experiments. Unpaired, two-tailed Student's *t*-test was used for analyses; **p < 0.01.

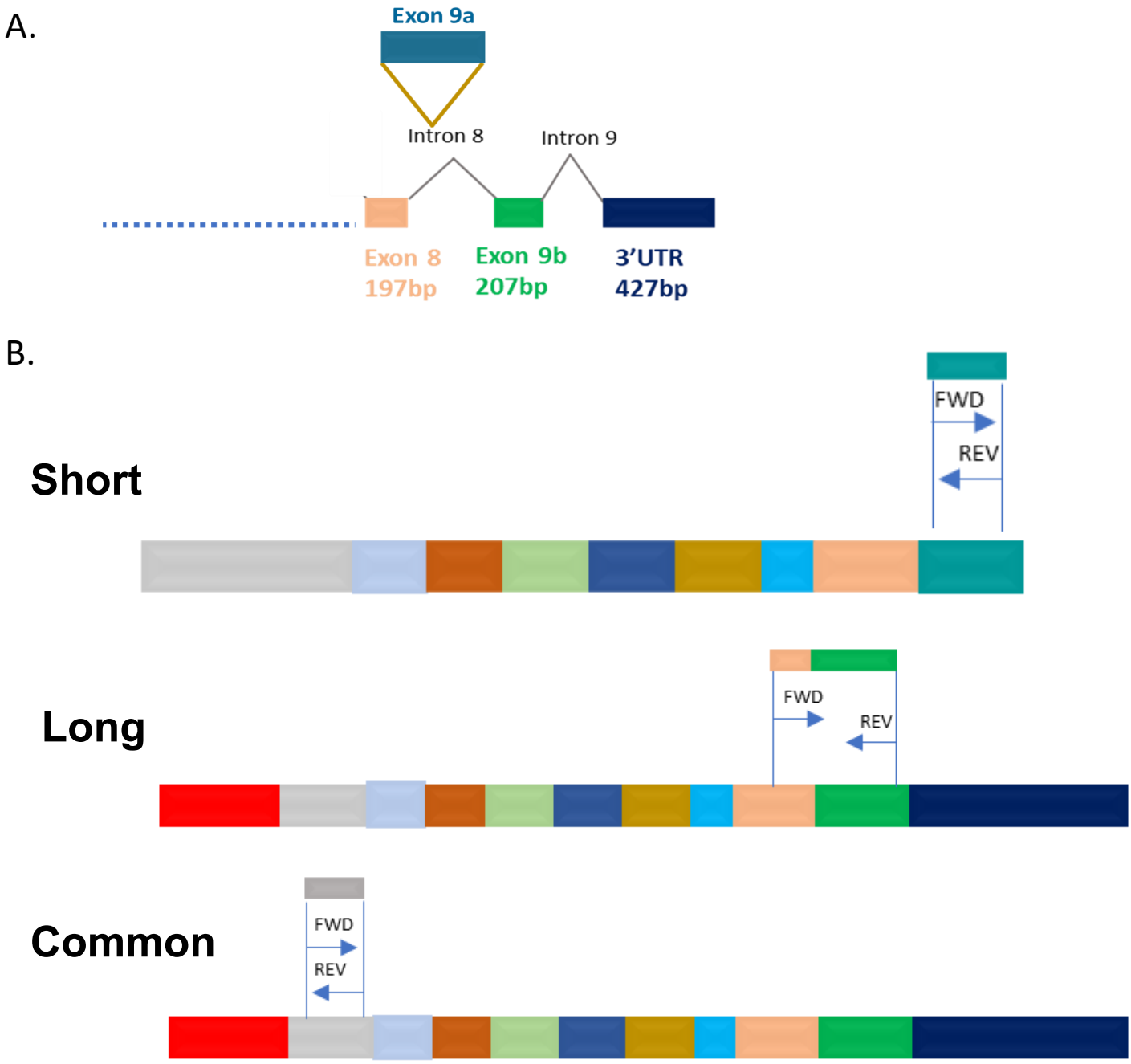

Figure 2.3 Gene structure of LKB1

(A) *Stk11*$_S$ is formed by the inclusion of exon9a instead of 9b. Exon 9a is a cryptic exon found in intron 8 (B) Schematic of the LKB1 primers used in this study.

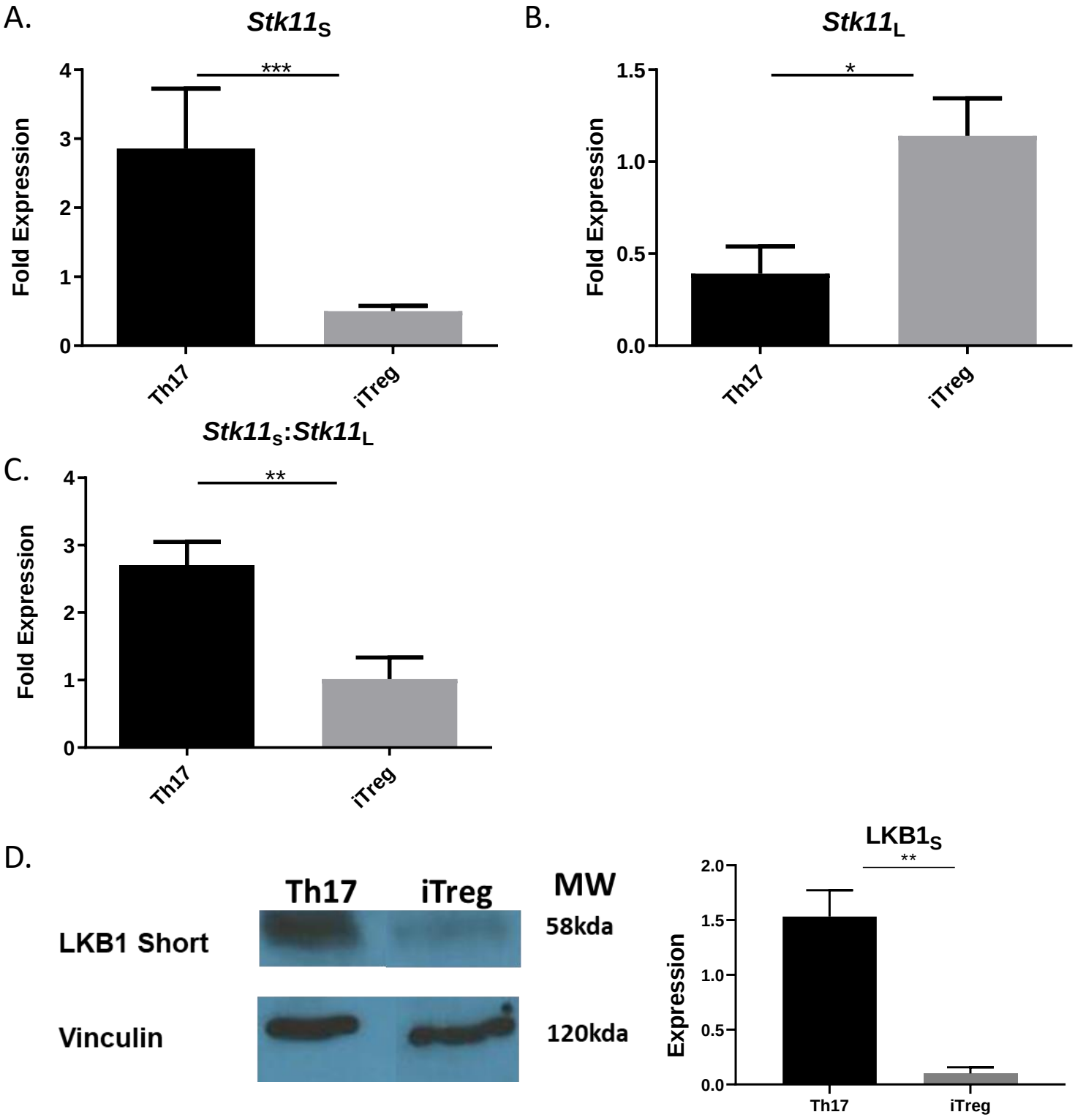

Figure 2.4 *Stk11*$_S$ is predominately expressed in Th17 cells

The splice variants of *Stk11* were quantified by qRT-PCR. The (A) short and (B) long splice variants, and the (C) relative short:long transcript ratio are shown. Relative gene expression was determined using the ΔΔCT method. The results are presented as the fold expression of the gene of interest normalized to the housekeeping gene β-actin (*ACTB*) for cells and relative to the NP sample. (D) The short isoform of LKB1 short was visualized by immunoblot, with vinculin as a loading control (left panel). Quantified protein expression is shown in the right panel. Data are the mean ± S.E.M of three independent experiments. Unpaired, two-tailed Student's *t*-test was used for analyses; *p < 0.05; **p < 0.01; ***p < 0.001.

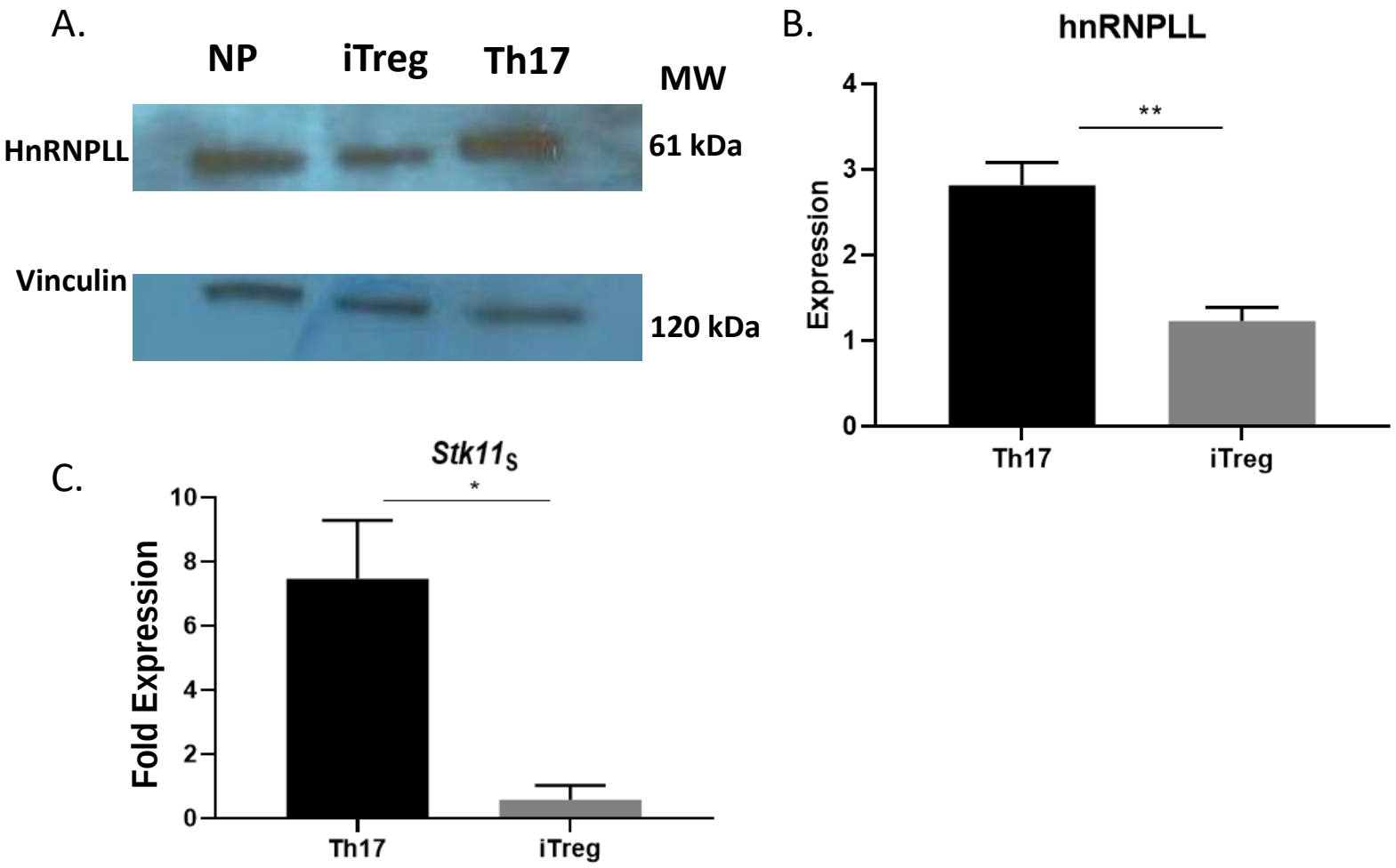

Figure 2.5 HnRNPLL binds *Stk11*$_S$ in Th17 cells

(A) HnRNPLL was visualized by immunoblot with vinculin as a loading control with (B) quantified protein expression. We used RNA-immunoprecipitation to quantify (C) Stk11$_S$ bound to hnRNPLL in Th17 and iTreg cells. Data was analyzed using the ΔΔCt method and values were normalized to input. Data are the mean ± S.E.M of three independent experiments. Unpaired, two-tailed Student's *t*-test was used for analyses; *p < 0.05; **p < 0.01; ***p < 0.001.

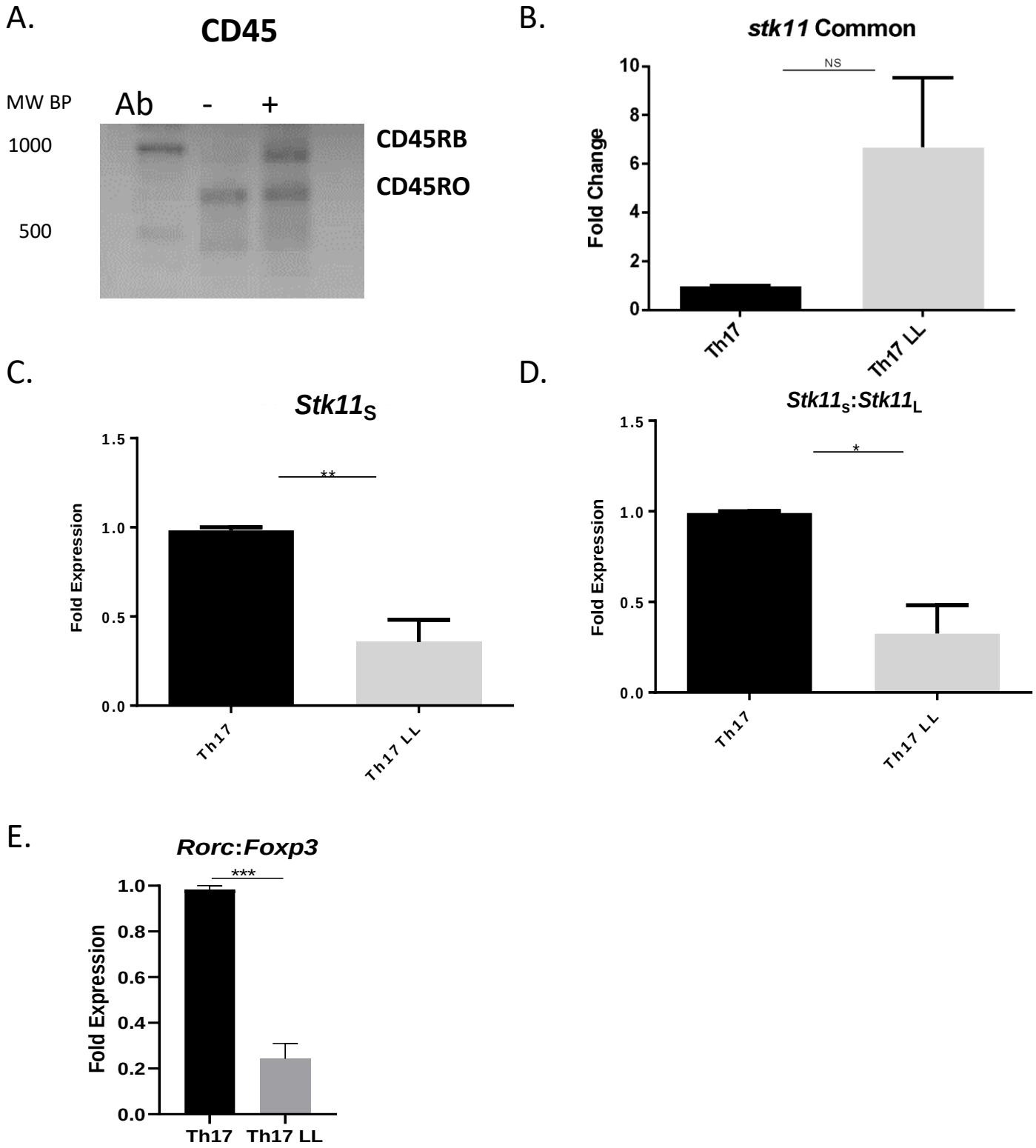

Figure 2.6 HnRNPLL regulates *Stk11*$_S$ splicing in Th17 cells

We delivered anti-hnRNPLL to Th17 cells and (A) verified its neutralizing effects by assessing CD45 splice variants in Th17 cells differentiated with or without anti-hnRNPLL. Following differentiation, we quantified (B) *Stk11*$_S$, (C) *Stk11*$_S$ (D) *Stk11*$_S$:*Stk11*$_L$, and (E), *Rorc*:*Foxp3* for Th17 cells left untreated or treated with anti-hnRNPLL. *Stk11*$_S$ and *Stk11*$_L$ levels were normalized to β-actin and are shown relative to the *Stk11* common primer to account for differences in transcription efficiency between treated and untreated cells. Relative gene expression was determined using the ΔΔCt method. Data are the mean ± S.E.M of three independent experiments. Unpaired, two-tailed Student's *t*-test was used for analyses; *p < 0.05; **p < 0.01; ***p < 0.001.

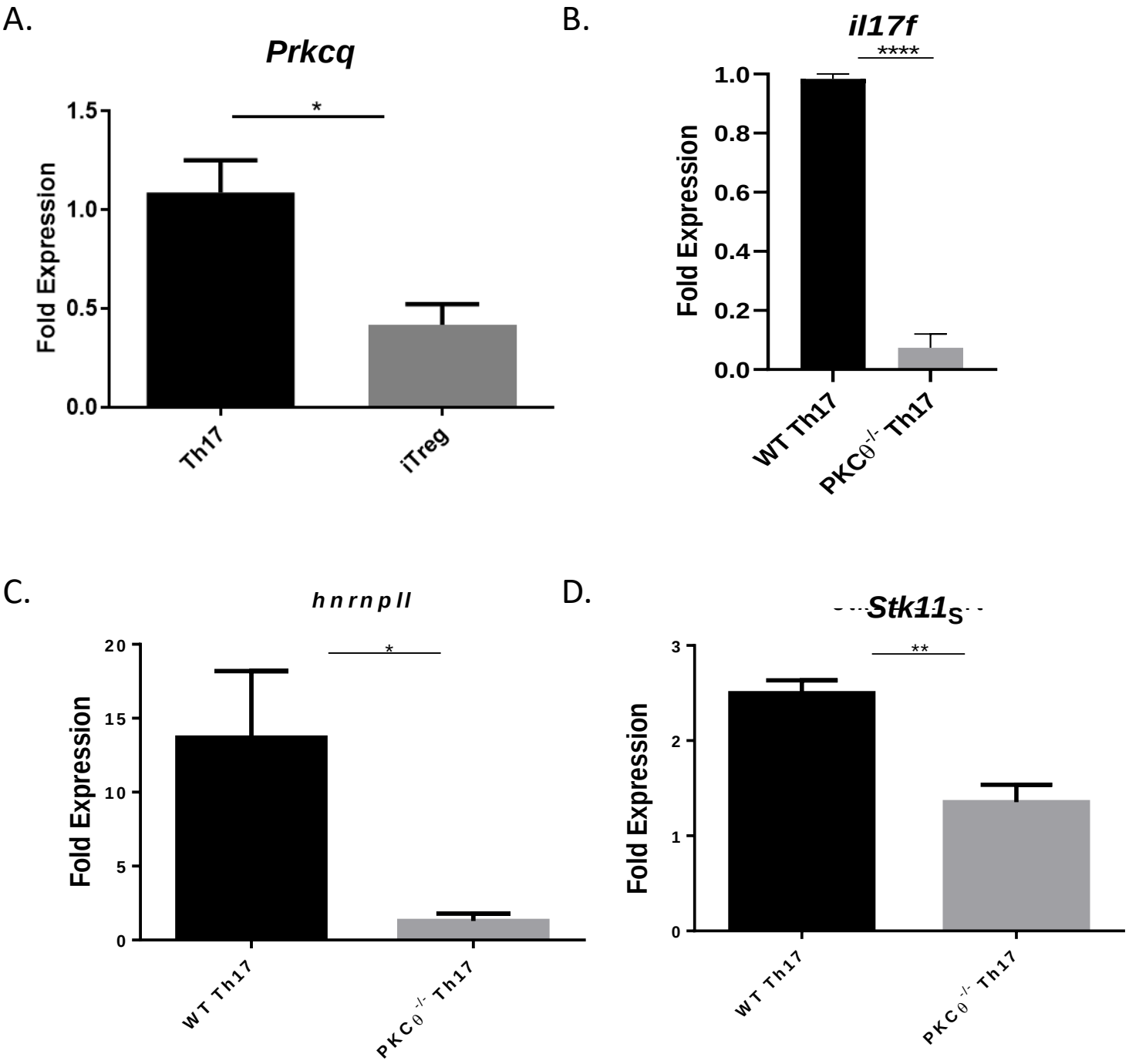

Figure 2.7 PKCθ regulates *Stk11*$_S$ expression

Prkcq expression was determined for (A) Th17 and iTreg cells. To assess the necessity of PKCθ in LKB1 splicing, WT and PKCθ-/- CD4 T cells were differentiated into NP or Th17 cells. qRT-PCR was used to quantify the expression of (B) *Il17f* , (C) *Hnrnpll*, and (D) *Stk11*$_S$. Relative gene expression was determined using the ΔΔCt method. The results are presented as the fold expression of the gene of interest normalized to the housekeeping gene β-actin (*ACTB*) for cells and relative to the wild type NP sample. Data are the mean ± S.E.M of three independent experiments. Unpaired, two-tailed Student's t-test was used for analyses; NS $p>0.05$ *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p<0.0001$.

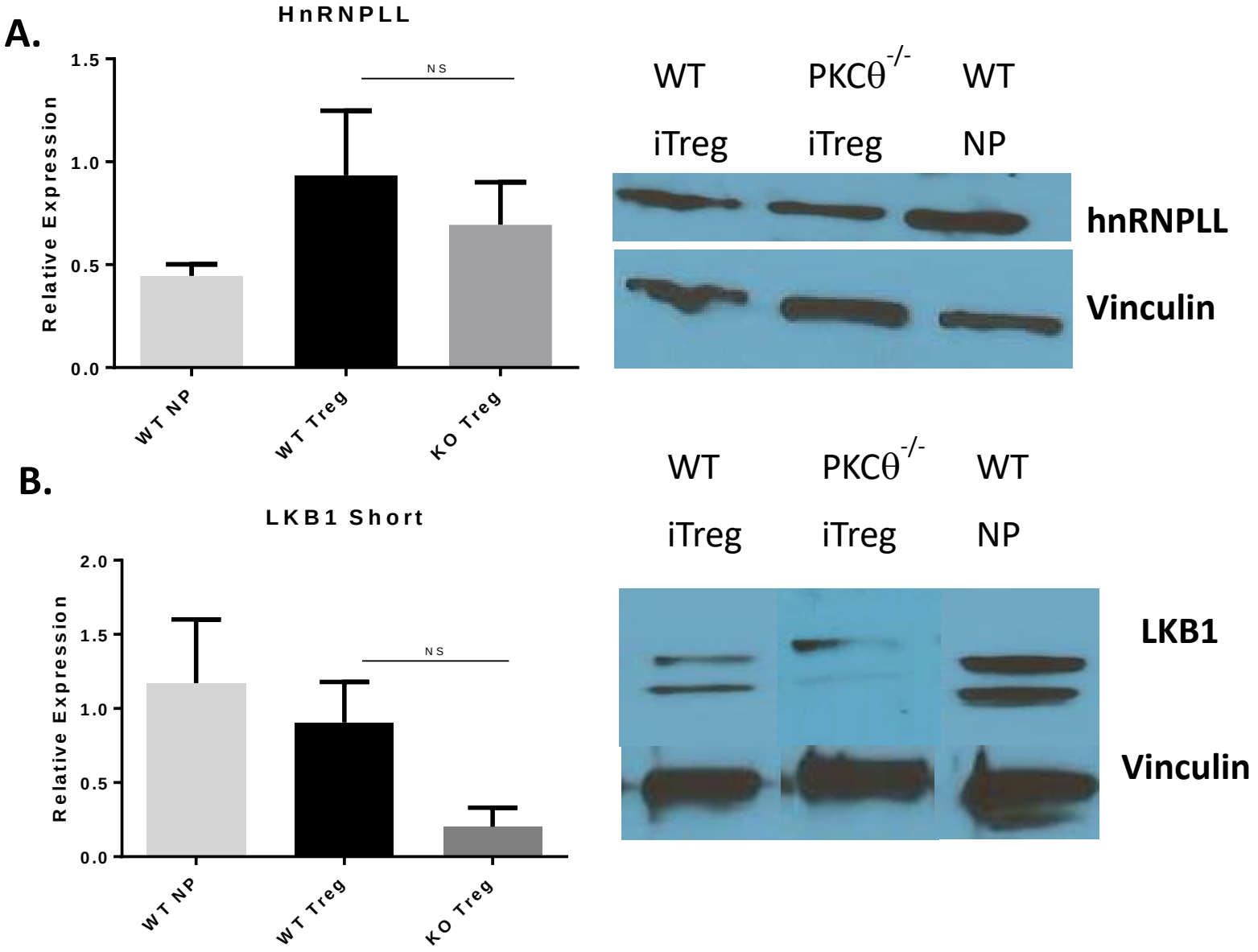

Figure 2.8 PKCθ regulates hnRNPLL and LKB1 expression in iTregs

WT and PKCθ-/- CD4 T cells were differentiated into NP or iTreg cells. The expression of (a) hnRNPLL and (B) LKB1 was measured by immunoblot with vinculin as a loading control. Data are the mean ± S.E.M of three independent experiments. Unpaired, two-tailed Student's t-test was used for analyses; NS $p>0.05$

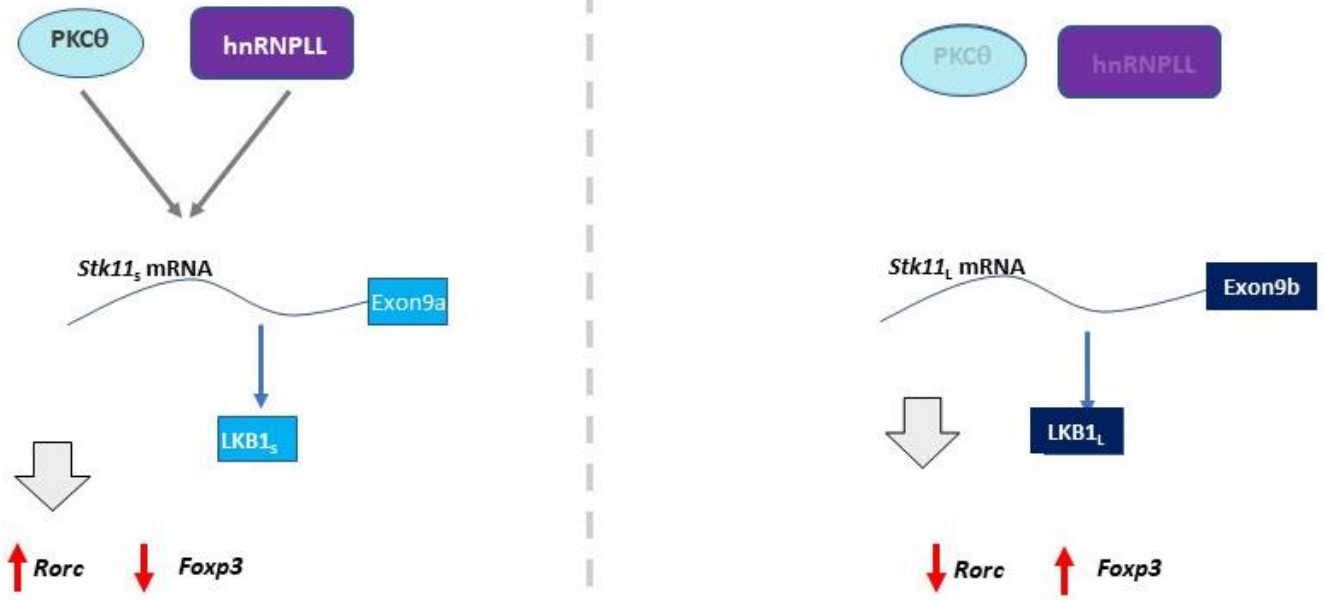

Figure 2.9 Model of PKCθ and hnRNPLL regulation of *Stk11* splicing

Th17 cells express more $Stk11_S$ than iTregs. In Th17 cells, hnRNPLL regulates the splicing of $Stk11_S$.Inhibition of *Stk11* reduces the amount of *Rorc:Foxp3*. In the absence of PKCθ, hnRNPLL and $Stk11_S$ expression is diminished.

CHAPTER 3

IL-6 SIGNALING REGULATES TH17-iTREG PLASTICITY AND METABOLISM THROUGH ITS REGULATION OF LKB1$_S$ AND PKCθ EXPRESSION

3.1 Introduction

The most striking difference between iTreg and Th17 cells lies in their distinct metabolic phenotypes. Th17 cells exhibit the most glycolytic phenotype of all the T helper subsets, while iTreg cells rely on energy processes that are vastly different from the metabolic phenotypes of all other T helper cells. Th17 cells utilize glycolysis, the pentose phosphate pathway, OXPHOS, the hexosamine pathway, and FAS, whereas iTreg cells engage catabolic processes, mainly FAO, to provide energy (Galgani et al., 2015). It is intriguing to think that T cells can go through such dramatic changes in metabolism following activation and yet retain the ability to alternate between anabolism and catabolism. Identifying regulators of metabolic status may lay the foundation for developing therapeutics to modulate the immune response.

We previously demonstrated that using rotenone to inhibit Complex I of the electron transport chain (ETC) and subsequent OXPHOS, we attenuated Th17 differentiation but not that of iTregs. Following rotenone treatment, Th17 cells upregulated *Foxp3* expression and exhibited reduced levels of *Rorc*, suggesting that these Th17 cells skew toward an iTreg phenotype when OXPHOS is perturbed (Ozay et al., 2018). It may seem counterintuitive for Th17 cells to lose “Th17-ness” by reducing *Rorc* and upregulating *Foxp3* expression, in the absence of OXPHOS. However, although Th17 cells are highly glycolytic cells, they still need OXPHOS for proper T cell

activation and proliferation consistent with the diminished *Rorc* in these cells. However, increased *Foxp3* upon Complex I inhibition is surprising as Complex I inactivity is correlated with Treg suppressive capacity (Angelin et al., 2016). Additionally, Foxp3 expression is important for driving OXPHOS through enhanced expression of OXPHOS enzymes important in this metabolic pathway (Howie et al., 2017).

A critical branchpoint between Treg and Th17 metabolic phenotypes rests at the induction of pyruvate oxidation. Glycolysis will induce the conversion of glucose to pyruvate. The pyruvate can be converted either into lactate and excreted from the cell or converted to acetyl-coA to feed the TCA cycle, through a process of pyruvate oxidation mediated by pyruvate dehydrogenase (PDH-e1α). Th17 cells resist pyruvate oxidation through the actions of pyruvate dehydrogenase kinase 1 (PDHK1), which will phosphorylate and inhibit PDH-e1α. PDHK1 activity is important for Th17 effector function because when it is inhibited in Th17 cells, these cells display a reduced effector phenotype, characterized by decreased IL-17a expression (Gerriets et al., 2015).

We further explored whether the diminished Th17 phenotype that we observed following rotenone treatment was due to reduced PDHK1 activity. Using imaging flow cytometry, we demonstrated that rotenone abrogates the mitochondrial localization of PDHK1 and PDH-e1α. We noted a similar effect when we treated cells with dichloroacetate (DCA), which also inhibits PDHK1 (Ozay et al., 2018).

Metabolism and plasticity are closely linked and several groups have demonstrated that perturbing metabolic pathways through pharmacological manipulation can affect T cell phenotype (Gualdoni et al., 2016; Ozay et al., 2018; Wang et al., 2016). This effect has been extensively studied in Th17-iTreg plasticity. It has been shown, *in*

vitro and *in vivo,* that Th17 cells can be reprogrammed into FOXP3+ T cells, and increased percentages of these cells correlated with increased disease survival in the presence of metabolic inhibitors such as metformin and (aminooxy)acetic acid. As such, understanding the mechanisms that mediate this plasticity will aid in developing therapeutics to modulate the immune responses by modulating metabolism. Specifically, in the case of autoimmunity, iTregs are severely compromised either in number or in function, or both. Identifying mediators of iTreg stability that preserve suppressive capacity could help treat autoimmune disorders (Ren and Li, 2017; Sun et al., 2017; Zhou et al., 2009). It is very peculiar that iTreg and Th17 cells can exhibit plasticity *in vitro* and *in vivo* especially considering the very different functions of these two cell types. Even more striking is that these two cell types differ dramatically in their metabolic phenotypes.

We noted distinct differences in the expression of the metabolic regulator LKB1 in Th17 and iTreg cells; therefore, we sought to determine whether isoform expression correlates with plasticity.

3.2 Results

3.2.1 LKB1 splicing machinery functions downstream of IL-6 signaling

The levels of *Stk11*$_S$ differ between Th17 and iTreg cells and, as such we postulated that this difference may correlate with the intrinsic plasticity observed between these two subsets. IL-6 signaling is key to Th17 cell differentiation and IL-6 deficient mice are unable to generate Th17 cells (Nish et al., 2014). Interestingly, IL-6 is strongly inhibitory towards iTreg differentiation, acting to block *Foxp3* expression (Samanta et al., 2008). Additionally, Th17 cells can transdifferentiate into RORγt^{lo}-Foxp3+-IL-10 expressing cells *in vivo* (Galgani et al., 2015). We hypothesized that

LKB1$_S$ is a signature feature of Th17 cell identity and not of iTregs. When we differentiated Th17 cells with anti-hnRNPLL treatment, we saw a reduced ratio of *Rorc*:*Foxp3* transcript, compared to Th17 cells differentiated with no treatment, Figure 2.6. These results suggested that hnRNPLL may function in modulating Th17-iTreg plasticity.

The pro-inflammatory cytokine, IL-6, promotes Th17 cell differentiation, *in vitro* and *in vivo*, and IL-6 receptor deficient mice are unable to generate Th17 cells (Morishima et al., 2009; Nish et al., 2014). IL-6 signals through Janus Kinase (JAK) and signal transducers and activators of transcription (STAT)3. As such, IL-6 acts to regulate transcription of *Rorc*, which encodes the Th17 master transcriptional regulator, RORγt, to drive Th17 differentiation (Zhou et al., 2009). In some *in vivo* models of colitis, which are accompanied by high levels of IL-6 expression, Foxp3+ Tregs upregulated *Rorc*, suggesting that IL-6 is a key regulator of plasticity between these subsets (Ren and Li, 2017). Collectively, these observations led us to hypothesize that IL-6 and PKCθ may synergize to regulate LKB1 splicing through hnRNPLL. We added IL-6 to iTregs on day 5 of differentiation, at the same concentration used for Th17 cell polarization. After 24 hours of culture with IL-6, iTregs upregulated hnRNPLL expression, Figure 3.1, leading to the intriguing possibility that there might also be changes in *Stk11* splicing. Indeed, in differentiating iTregs cultured with IL-6, we observed increased *Stk11*$_S$, Figure 3.1. We also detected elevated levels of *Rorc* transcript, Figure 3.1, indicating acquisition of a Th17-like phenotype.

3.2.2 PKCθ expression is regulated by IL-6 signaling

Our data indicated that PKCθ regulated the expression of hnRNPLL in Th17 cells, (Figure 2.7), and that IL-6 signaling upregulated hnRNPLL in iTregs, (Figure 3.1). Furthermore, it has been shown that IL-6 and PKCθ activity can both destabilize the Treg phenotype. Since we observed IL-6 and PKCθ regulated hnRNPLL expression and, consequently, *Stk11* splicing, we hypothesized that IL-6 signaling might also induce PKCθ expression. As we predicted, when iTregs were treated with IL-6, *Prkcq,* the gene encoding PKCθ, was strongly upregulated, Figure 3.2. This indicates that T cells respond to the presence of IL-6 by upregulating *Prkcq.* Collectively, these data support an outside-in signaling pathway whereby iTregs respond to the presence of IL-6 by upregulating *Prkcq,* re-expressing hnRNPLL, and generating higher levels of *Stk11*s to facilitate iTreg to Th17 plasticity.

We were intrigued by this finding because the only previously reported connection between IL-6 and PKCθ was through PKCθ-mediated STAT3 upregulation in Th17 cells. We hypothesized that this mechanism may proceed, in part, through the upregulation of hnRNPLL, downstream of IL-6 signaling. Using the RBPmap algorithm, we examined the *Prkcq* sequence for hnRNPLL binding sites. We were surprised to find numerous potential hnRNPLL binding sites in the sequence. We performed RNA-immunoprecipitation, as previously described, immunoprecipitating transcripts bound to hnRNPLL. Using qRT-PCR, we established that hnRNPLL binds to *Prkcq* in Th17 cells but not in iTreg cells, Figure 3.2. These data suggest, in fully differentiated iTregs, that IL-6 signaling upregulates hnRNPLL and, subsequently, induces the expression of PKCθ. These novel observations provide some insight as to how IL-6 negatively

regulates Treg programming through its effects on hnRNPLL and PKCθ, culminating in alterations of *Stk11*s expression levels.

3.2.3 Pharmacological perturbation of lactate production reduces *Stk11*s

The LKB1-AMPK pathway is best known for functioning as a metabolic sensor, shifting cells towards energy preservation pathways to effectively block anabolic pathways such as glycolysis, and promoting catabolic pathways such as fatty acid oxidation (Lochner et al., 2015). Th17 cells are highly glycolytic; therefore, we found it surprising that these cells expressed such high levels of LKB1. Further, perturbating metabolism has been shown to modulate T cell phenotypes (Ren and Li, 2017). As such, we hypothesized that hnRNPLL promotes *Stk11* alternative splicing in Th17 cells, to prevent changes in metabolism, and the effects of *Stk11* splicing following anti-hnRNPLL delivery supports this notion, Figure 3.1. When T cells are activated, they utilize glycolysis to convert glucose to pyruvate which will then be converted to lactate and excreted from the cell. This mode of energetics is also used by effector T cells after activation. Treg cells do not use glycolysis as a primary means of deriving energy. Instead of breaking-down glucose *via* glycolysis, Treg cells preferentially break down fatty acids and proteins to fuel the TCA cycle (Galgani et al., 2015).

On the infrequent occasion that glycolysis occurs in Tregs, the pyruvate generated will be converted to acetyl-coA to feed into the TCA cycle. Pyruvate is processed into acetyl-coA through the actions of PDH. PDH activity is regulated by PDHK1 which will phosphorylate and inactivate PDH, allowing pyruvate to be converted into lactate and excreted from the cell. Th17 cells express higher levels of PDHK1 than do Tregs or even Th1 cells. Altogether this supports a model whereby Th17 cells are highly reliant on glycolysis, and concomitant lactate production. As such, inhibition of acetyl-coA production is a fundamental feature of Th17 biology (Gerriets et al, 2015).

We hypothesized that LKB1$_S$ may be generated as a mechanism to maintain glycolysis. To test this, we differentiated Th17 cells and treated them during the last 48 hours of differentiation with dichloroacetate (DCA) which inhibits glycolysis by inhibiting pyruvate dehydrogenase kinase (PDHK), a kinase that blocks pyruvate oxidation (Gerriets et al., 2015). We validated that DCA treatment inhibited glycolysis by assessing transcript levels of hexokinase 2 (*HK2)*, an enzyme required for glycolysis, Figure 3.3. Following DCA treatment, *Stk11*$_S$ transcript was abrogated while *Stk11*$_L$ remained relatively constant, Figure 3.3. Additionally, Th17 cells treated with DCA showed a markedly decreased ratio of *Stk11*$_S$:*Stk11*$_L$, Figure 3.3, consistent with the results we observed when we delivered anti-hnRNPLL, Figure 2.6. These experiments demonstrate for the first time that LKB1 isoform expression is associated with, and changes with, the metabolic profile of the Th17 cells.

When *Stk11*$_S$ alternative splicing was attenuated, the ratio of *Rorc*:*Foxp3* was lowered concomitantly, Figure 2.6, suggesting these cells were adopting an iTreg-like phenotype. Given its effects on *Stk11* splicing, we asked whether DCA treatment would also destabilize the Th17 phenotype. We noted diminished expression levels of *Rorc* in DCA-treated Th17 cells, Figure 3.4, thus linking Stk11 splicing and plasticity to metabolic status.

Futhermore, we observed that *Prkcq* was induced by upstream IL-6 signaling and that this correlated with hnRNPLL expression, Figure 3.1. Additionally, we demonstrated that both *Stk11*$_S$ and *Prkcq* transcripts were enriched in hnRNPLL-immunoprecipitated Th17 lysates (Figure 2.5 and 3.2). Therefore, we asked whether *Prkcq* expression would be impacted similarly to that of *Stk11*$_S$ when glycolysis is perturbed. We noted a significant reduction in *Prkcq* expression in Th17 cells, treated with DCA, Figure 3.4. Collectively, these data provide evidence of a highly integrated network that links metabolic state with *Rorc* and *Prkcq* expression, as well as with *Stk11* alternative splicing.

3.3 Discussion

In this study, we asked whether there was a relationship between *Stk11* splicing, plasticity and metabolism. We showed that IL-6 signaling upregulated hnRNPLL expression and subsequently increase *Stk11*$_S$ expression. We also demonstrated for the first time that IL-6 signaling acts upstream of and positively regulates PKCθ by increasing *Prkcq* transcription. To further tease out the mechanism of *Prkcq* upregulation, we asked if hnRNPLL, which is also induced by IL-6, modulated *Prkcq* transcription. We found by RNA-immunoprecipitation, that hnRNPLL binds to *Prkcq* transcript in Th17 cells but not in iTregs. Previous studies demonstrated IL-6 acts to inhibit Treg differentiation and function (Brezar et al., 2015; Zanin-Zhorov et al., 2010) and the data we provide linking IL-6 to *Prkcq* regulation fills a critical gap in our understanding of how IL-6 signaling acts to destabilize the Treg phenotype.

LKB1 functions in effector T cells have not been fully elucidated. In peripheral T cells, LKB1 depletion increased glycolysis, cell death, and cytokine production by Th1 and Th17 cells (MacIver et al., 2011). LKB1 has been shown to mediate fatty acid metabolism and this feature is critical for Treg function (Timilshina et al., 2019) and contrastingly act in pathways to inhibit glycolysis (Ma et al., 2017). As Th17 cells are highly glycolytic, our results beg the question whether the splicing of *Stk11* in Th17 cells is necessary to maintain glycolysis. This is based on our observation that when we used DCA to inhibit glycolysis in Th17 cells, we also attenuated *Stk11*$_S$ expression, highlighting a reciprocal relationship between *Stk11*$_S$ expression and maintaining glycolysis. Whether or not *Stk11* splicing defines the metabolic state of Th17 cells, remains to be elucidated. What we can conclude, however, is that IL-6 signaling promotes *Stk11*$_S$ transcription, and

this proceeds *via* PKCθ and hnRNPLL, Figure 3.5. Altogether, our data suggest that LKB1 splicing may be central to mediating iTreg-Th17 plasticity.

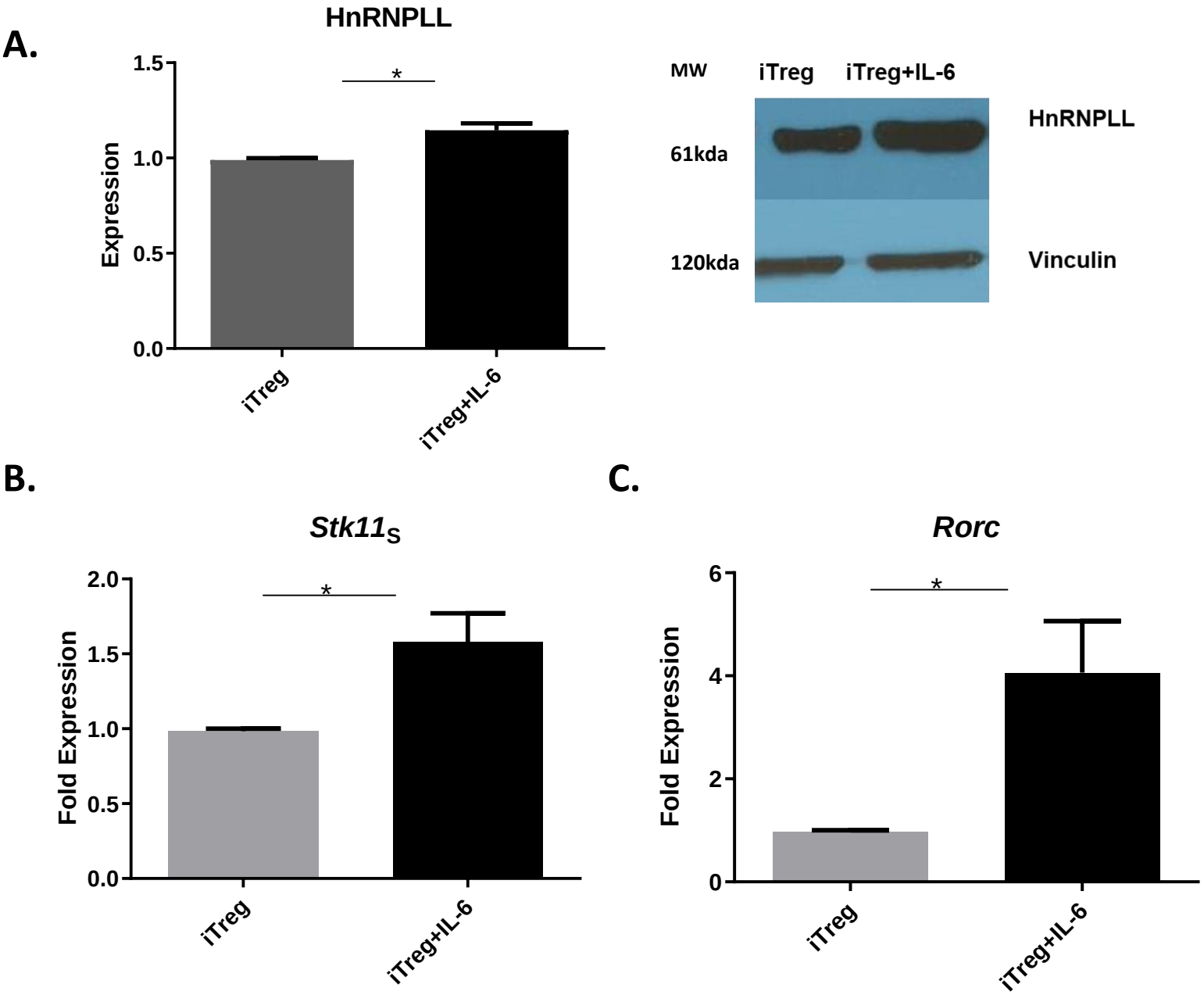

Figure 3.1 IL-6 signaling induces hnRNPLL and *Stk11*$_S$ expression in iTregs

iTregs were dosed with 20ng/mL of IL-6 of day 5 of the differentiation culture. Cells were harvested 24 hours later. We used immunoblotting to determine (A) hnRNPLL expression and used qRT-PCR to quantify (B) *Stk11*$_s$, (C) *Rorc.* Relative gene expression was determined using the ΔΔCt method. The results are presented as the fold expression of the gene of interest and normalized to β-actin (*ACTB*) and relative to the untreated iTreg sample. Data are the mean ± S.E.M of three independent experiments. Unpaired, two-tailed Student's *t*-test was used for analyses; *p < 0.05; **p < 0.01; ***p < 0.001.

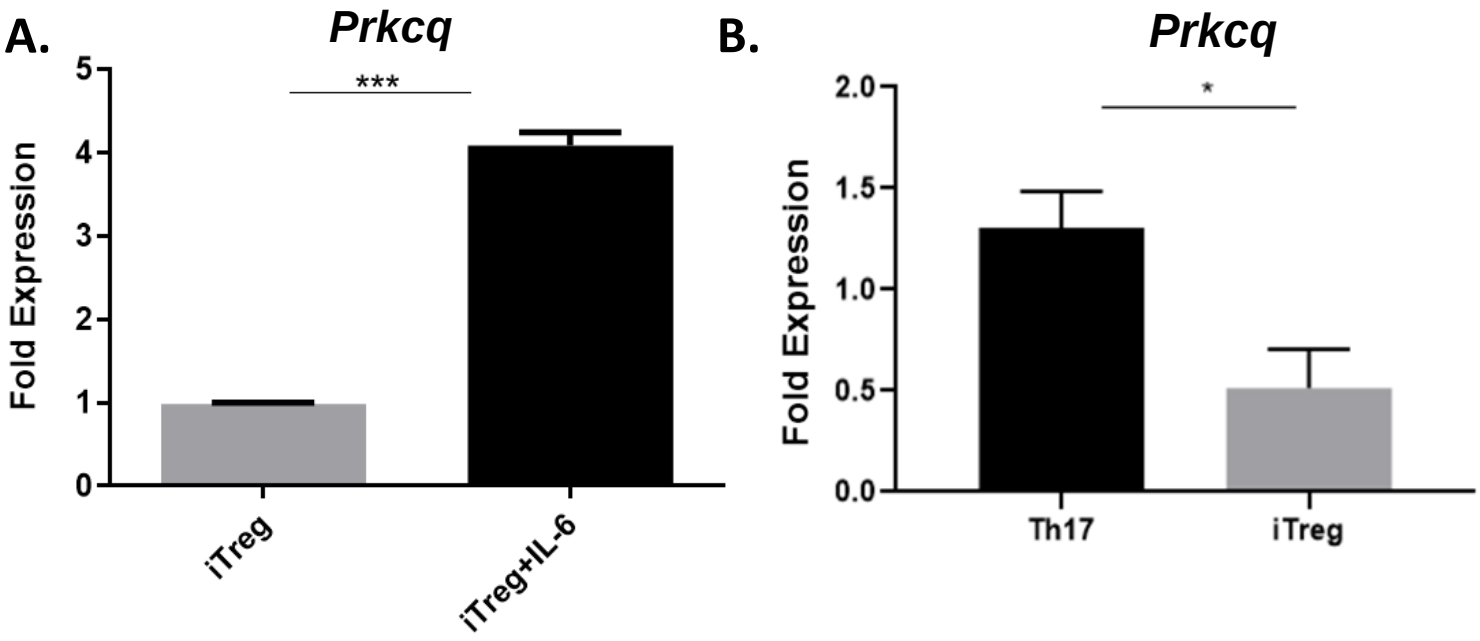

Figure 3.2 IL-6 and hnRNPLL regulate PKCθ expression

iTregs were dosed with 20ng/mL of IL-6 of day 5 of the differentiation culture. Cells were harvested 24 hours later. We used qRT-PCR to quantify *Prkcq* expression (A) and from transcripts immunoprecipitated to hnRNPLL (B). Relative gene expression was determined using the ΔΔCt method. The results are presented as the fold expression of the gene of interest and normalized to β-actin (ACTB) and relative to the untreated iTreg sample (A) or NP (B). Data are the mean ± S.E.M of three independent experiments. Unpaired, two-tailed Student's t-test was used for analyses; *$p < 0.05$; ***$p < 0.001$.

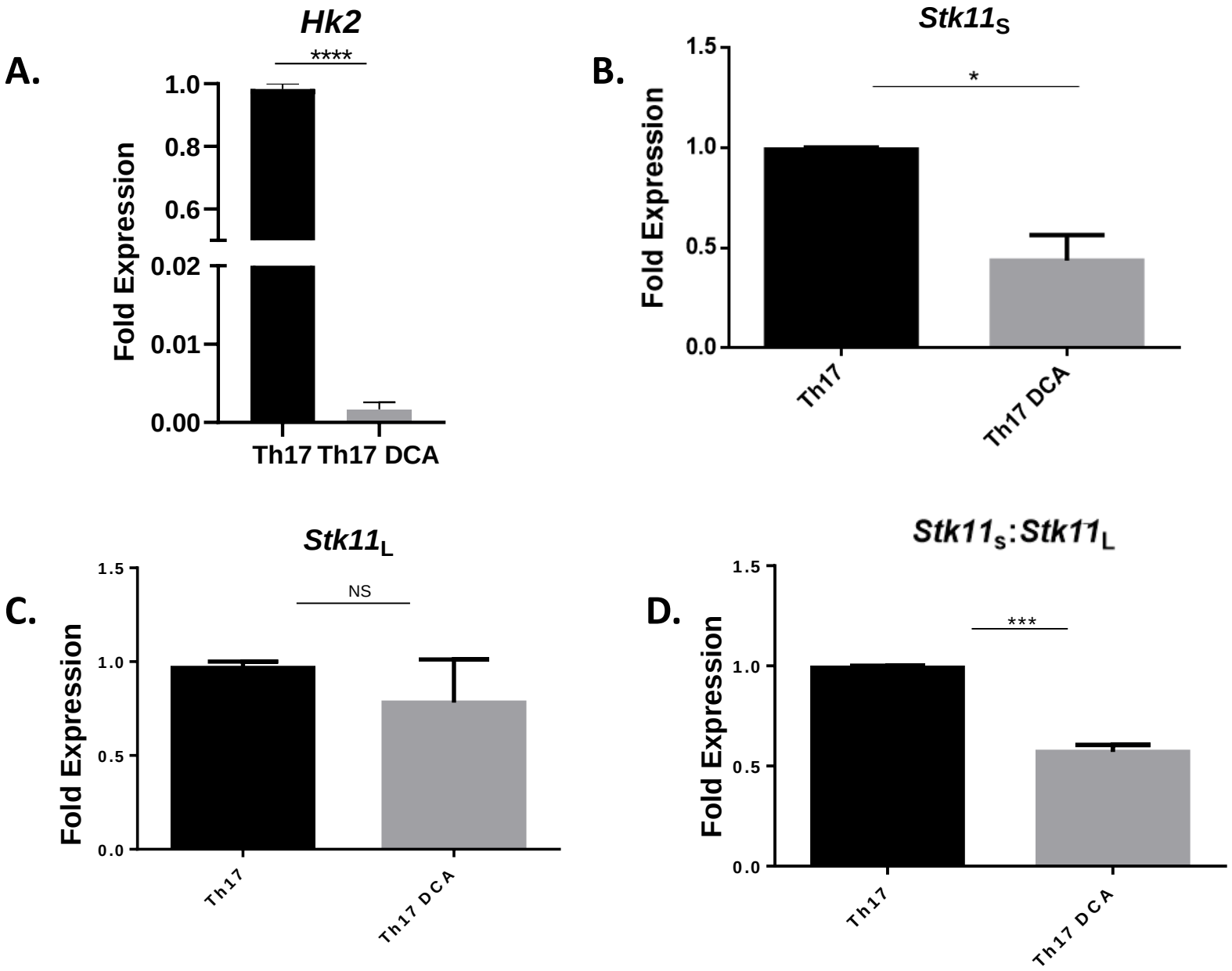

Figure 3.3 Modulating Th17 cell metabolism alters *Stk11*$_S$ expression

CD4 T cells were differentiated under Th17 polarizing conditions. During the last 48 hours of differentiation, Th17 cells were dosed with 10mM of dichloroacetate (DCA). DCA effects on glycolysis were confirmed by quantifying transcript of (A) Hexokinase2. We used qRT-PCR to assess the effects on DCA treatment on (B) *Stk11*$_S$, (C) *Stk11*$_L$, (D) *Stk11*$_S$:*Stk11*$_L$. Relative gene expression was determined using the ΔΔCt method. The results are presented as the fold expression of the gene of interest normalized to the housekeeping gene β-actin (*ACTB*) for cells and for (A) relative to the untreated Th17 sample and for (B-D) and relative to the common primer to account for differences in overall *Stk11* transcription levels. Data are the mean ± S.E.M of three replicates and are representative of three independent experiments, each of which showed similar results. Unpaired, two-tailed Student's t-test was used for analyses; *p < 0.05; ***p < 0.001; ****p<0.0001.

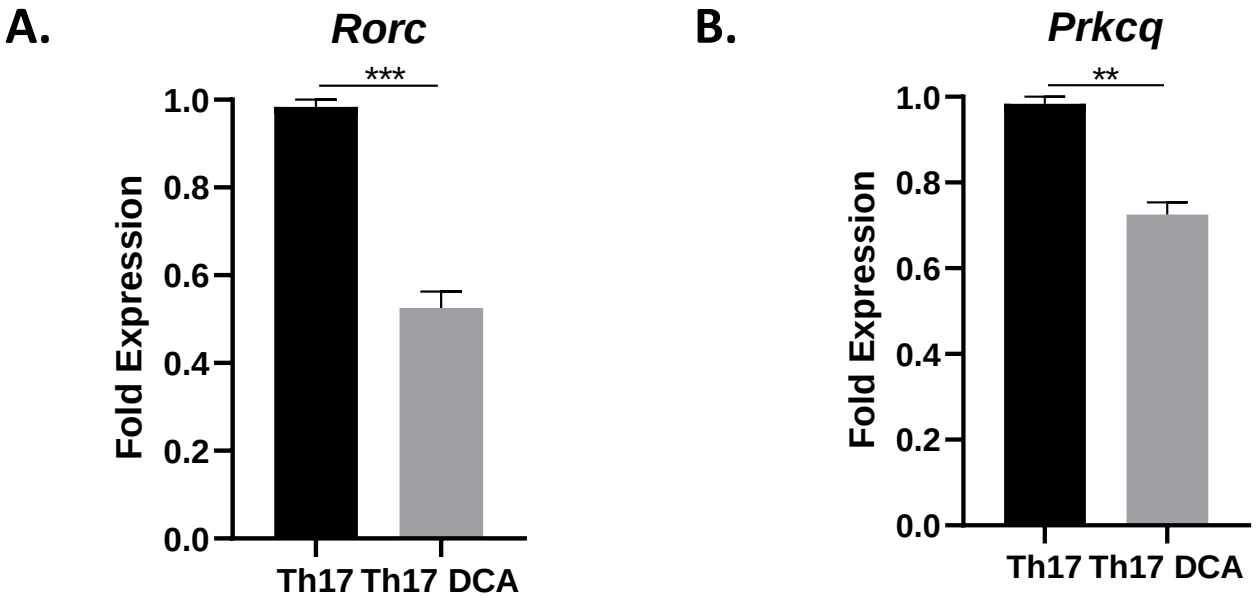

Figure 3.4 Modulating Th17 cell metabolism diminishes Th17 phenotype

CD4 T cells were differentiated under Th17 polarizing conditions. During the last 48 hours of differentiation, Th17 cells were dosed with 10mM of Dichloroacetate (DCA). We used qRT-PCR to assess the effects on DCA treatment on (A) *Rorc*, and (B) *Prkcq.* Relative gene expression was determined using the ΔΔCt method. The results are presented as the fold expression of the gene of interest normalized to the housekeeping gene β-actin (*ACTB*) for cells and for relative to the untreated Th17 sample. Data are the mean ± S.E.M of three replicates and are representative of three independent experiments, each of which showed similar results. Unpaired, two-tailed Student's t-test was used for analyses; $^{**}p < 0.01$; $^{***}p < 0.001$.

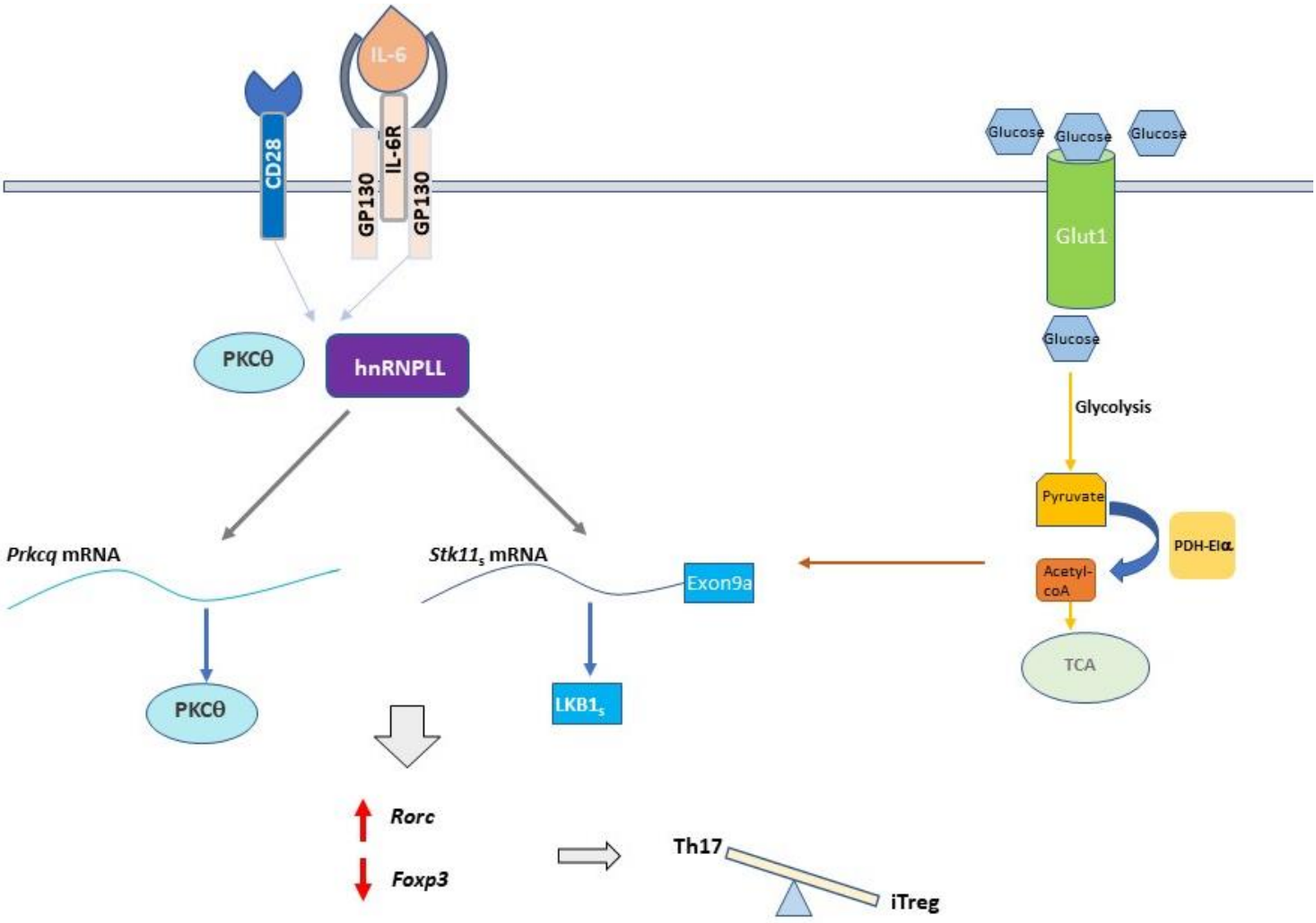

Figure 3.5 IL-6 signaling induces *Stk11*$_S$ and PKCθ expression

IL-6 signaling in combination with CD28 engagement induce the expression of *Prkcq* and hnRNPLL. hnRNPLL modulates the splicing of *Stk11*$_S$ and regulates *Prkcq* transcript. When hnRNPLL function is intact, there is more *Rorc* than *Foxp3* transcript in the cell, suggesting a Th17 phenotype. When glycolysis is inhibited *Stk11*$_S$, *Rorc*, and *Prkcq* expression is also inhibited. iTregs will yield a similar phenotype upon IL-6 stimulation to facilitate iTreg to Th17 plasticity.

CHAPTER 4

CONCLUSIONS AND FUTURE DIRECTIONS

4.1 Conclusions

In this study, we describe for the first time a connection between LKB1 isoform expression, metabolism, and plasticity between Th17 cells and iTregs. LKB1 has been identified as a mediator of Treg induction and function through its effects on TSDR methylation and on cell metabolism. We demonstrate that LKB1 is expressed in both Th17 and iTreg cells, and to a much higher extent in Th17 cells. LKB1 can be expressed as one of two isoforms: short and long, which differ in their expression of exon 9. We have determined that $LKB1_S$ is predominately expressed in Th17 cells and $LKB1_L$ is expressed more abundantly in iTreg cells. We provide evidence that the isoform expression correlates with the phenotypic stability observed between Th17 cells and iTregs. As such, when IL-6, which is inhibits the Treg differentiation programming, is administered to iTregs, we observe increased levels of *Stk11*$_S$. Moreover, we demonstrate that IL-6 also upregulates PKCθ in iTregs and expression of PKCθ correlates with $LKB1_S$ expression. Finally, we provide evidence that LKB1 isoforms also correlate with the metabolic state of Th cells, because when we inhibit glycolysis, we see concomitant decrease in LKB1s levels in Th17 cells. This work reports, for the first time, data linking IL-6, PKCθ, and metabolic changes to differences in LKB1 isoform expression as being functionally relevant contributors to the plasticity of Th17 cells and iTregs.

4.2 Future Directions

4.2.1 HnRNPL and *Stk11* regulation

Heterogenous nuclear ribonuclear protein L (hnRNPL) shares sequence similarity to hnRNPLL and both proteins preferentially bind to dinucleotide CA repeats. According to the RBPmap algorithm, hnRNPL also demonstrates strong binding potential to *Stk11* transcript. Additionally, hnRNPL has been shown to inhibit cryptic exon inclusion. As such, we hypothesized that hnRNPL may block the splicing of *Stk11* short by blocking exon9a which is a cryptic exon, Figure 2.3, and this could account for the differences in *Stk11* isoforms seen between Th17 and iTreg cells. We hypothesized that perhaps there may be differences in hnRNPL binding to *Stk11* transcript in Th17 *versus* iTreg cells. Specifically, we expected to see more binding of hnRNPL to *Stk11*$_L$, as this interaction would be increased. We performed RNA-immunoprecipitation using anti-hnRNPL to immunoprecipitate target transcripts. Using qRT-PCR as an output, we observed that hnRNPL did bind to *Stk11*$_S$ and this was more prevalent in Th17 cells than in iTregs. Furthermore, hnRNPL binding to *Stk11*$_L$ was detectable both in Th17 and iTreg cells, Figure 4.1. However, it remains unclear whether hnRNPL binding blocks or drives the splicing of *Stk11*. To ask this question, we will need to conduct an antibody delivery experiment to hnRNPL, as done in Figure 2.6 and assess what happens to *Stk11* transcript when hnRNPL is functionally inhibited. If there are higher levels of *Stk11*$_S$, we can postulate that hnRNPL inhibits *Stk11* splicing.

Since we demonstrated more RNA binding, as well as increased *Prkcq* transcript, in Th17 cells we hypothesized that PKCθ may regulate the expression of *hnrnpl.*

However, we did not find a significant different in *hnrnpl* transcript expression WT and PKCθ$^{-/-}$ Th17 cells.

We next asked if, hnRNPL bind to PKCθ and, thus, might hnRNPL regulation proceed through its physical interaction? When we immunoprecipitated hnRNPL, we noted that it associated with PKCθ both in Th17 and iTreg cells, Figure 4.1, suggesting preferential binding may not regulate hnRNPL expression.

In Ozay et al. (2020) our group determined that nuclear hnRNPL is diminished in human iTreg cells treated with anti-phospho-PKCθ(T538), delivered intracellularly, while the cytosolic localization of hnRNPL is increased. This suggests that PKCθ may function in hnRNPL cytoplasmic-nuclear shuttling. HnRNPL is one of the hnRNP proteins that can shuttle and aids in cytoplasmic transcript accumulation (Kim et al., 2000). As PKCθ seems to be a regulator to Th17-iTreg plasticity, we hypothesized that hnRNPL localization would be primarily cytosolic in the absence of PKCθ in Th17 cells. We examined cytoplasmic and nuclear extracts from WT and PKCθ$^{-/-}$ Th17 cells and observed that nuclear hnRNPL was higher in PKCθ$^{-/-}$ Th17 cells as compared to WT Th17 cells, suggesting that PKCθ may act to regulate hnRNPL transport out of the nucleus, Figure 4.1. If hnRNPL is indeed inhibitory to *Stk11*$_S$ expression, this finding could further explain why there is less *Stk11*$_S$ expression in the absence of PKCθ. However, this data is preliminary and needs to be further validated.

Additional studies are needed to further tease out how exactly PKCθ regulates hnRNPL cellular localization. Since we observe a similar phenotype in iTreg cells and PKCθ$^{-/-}$ Th17 cells in regards to *Stk11* expression, we can postulate that what we see in the PKCθ$^{-/-}$ Th17 would be similar to WT iTregs. However, since we noted that hnRNPL

can bind *Stk11*$_S$, the isoform which is more highly expressed in Th17 cells than in iTregs or in PKC$\theta^{-/-}$ Th17 cells, there is much we have yet to learn about how hnRNPL functions in *Stk11* splicing.

If hnRNPL does have a role in regulating *Stk11*, it will be useful to determine if hnRNPL acts in the cytoplasm or in the nucleus. This can be answered, in part, by the antibody delivery experiment already mention and RNA-immunoprecipitation to further validate the binding of hnRNPL to transcript. We can then determine where this interaction occurs by separating cytoplasmic and nuclear RNA. Amplifying each cellular RNA fraction using qRT-PCR, we can determine whether this interaction is primarily cytosolic or nuclear. We can also perform this experiment using PKC$\theta^{-/-}$ Th17 cells, to determine whether this is a PKCθ-mediated phenomenon.

4.2.2 LKB1-Notch1 interactions in Th17 plasticity

In cardiomyocytes, LKB1 can interact with Notch1 which enables LKB1 to activate AMPK (Yang et al., 2016). Notch1 is a transmembrane protein that is activated by one of several ligands and then is sequentially cleaved by ADAM proteases and gamma-secretase. Notch1 has many different roles in T cell activation and differentiation (Osborne and Minter, 2007). Our group has shown that Notch1 interacts with PKCθ in the lipid rafts and this interaction functions to drive the activation of the CARMA1-BCL10-MALT1 complex and resultant NF-κB activation (Shin et al., 2014). However, in *ex vivo*-expanded cord blood Treg cells, PKCθ was found to be sequestered away from the immunological synapse upon T cell activation. Additionally, inhibition of PKCθ led to the increased suppressive activity of these Treg cells (Zanin-Zhorov et al., 2010).

LKB1 has been shown by numerous studies to be imperative to Treg survival and suppression (He et al., 2017; Timilshina et al., 2019; Wu et al., 2017; Yang et al., 2017). As such, we hypothesized that with the absence of PKCθ in the lipid rafts, Notch1 would preferentially bind to LKB1 in iTreg cells, whereas in effector cells, such as Th17 cells, Notch1 would preferentially bind to PKCθ. In order to investigate this hypothesis, we stained iTreg and Th17 cells with antibodies specific for Notch1 and LKB1, then utilized stochastic optical reconstruction microscopy (STORM) to visualize differences in protein localization between the two cell types. Surprisingly, we saw that there were more overlapping regions in the Th17 cells as compared to the iTregs. Using a colocalization algorithm, we determined that Th17 cells have a higher degree of colocalization as compared to the Treg cells, Figure 4.2. We will follow up this preliminary experiment using an immunoprecipitation assay to confirm the microscopy data. If we can confirm the microscopy data, the following work should provide a means of assessing the functional output of this interaction.

Our hypothesis is that the interaction between Notch1 and LKB1 may be tissue-context specific and thus in the Th17 cells, we predict Notch1-LKB1 interaction may modulate AMPK activity in a cell-type-specific manner. Previously, we established that Notch1 is associated with mitochondria more in iTregs than Th17 cells (Ozay et al., 2018). LKB1 has been reported to only reside in the nucleus or the cytoplasm (Zhu et al., 2009). As such, we can hypothesize that the differences in binding are due to protein accessibility of Notch1. We saw that the levels of nuclear LKB1 were relatively consistent both in Th17 cells and in iTregs, so we would predict that differences in

LKB1-Notch1 interaction may be because Notch1 accessibility is rate-limiting, Figure 2.2.

Interestingly, despite the inhibitory effect of AMPK on mTORC1, AMPK is essential, *in vivo*, for Th17 function (Blagih et al., 2015). However, one of the functions of AMPK is to inhibit the FAS enzyme, acetyl-CoA carboxylase 1, ACC1, which is imperative to Th17 development and acts to inhibit Treg development (Berod et al., 2014). Moreover, inhibition of AMPK or FAS can induce interferon γ (IFNγ) expression (Blagih et al., 2015; Young et al., 2017). This suggests that inhibiting fatty acid synthesis may regulate the transition of a Th17 cell into a pathogenic Th1-like phenotype, which are known to contribute to autoimmune diseases, such as multiple sclerosis (Young et al., 2017). Although it is unknown if Notch1 or LKB1 regulates the transition of a Th17 cell into a pathogenic Th-like cell. We hypothesize that a basal level of Notch1-LKB1 colocalization in the Th17 cells, as compared to iTregs, would position Th17 cells to readily adopt this more pathogenic phenotype.

To test these hypotheses after the initial confirmation by immunoprecipitation of interaction differences between the two cell types, we would want to explore the functional characteristics of this interaction. First, we can validate a tool to disrupt the interaction between Notch1 and LKB1 in Th17 cells. If this interaction relies on activated Notch1, we can use a gamma secretase inhibitor (GSI), to block Notch1 activation and use this as a tool to block the interaction. First, we will examine the activation of AMPK resultant activation of p-ACC1 by immunoblot in the presence or absence of GSI. If we observe changes in AMPK and p-ACC1 levels when Notch1 is inhibited, we can conclude that Notch1 activation is necessary for downstream of AMPK and p-ACC1

activity. Next, we can ask whether Th17 cells, treated with IL-23 and/or IL-1β to induce a pathogenic phenotype, have increased p-ACC1 and p-AMPK, as assessed by western blot? Further, if the Notch1-LKB1 interaction is important in FAS and resultant pathogenicity of Th17 cells, we would expect that upon GSI treatment of the pathogenic Th-like cells, there would be a diminution in the expression levels of p-ACC1 and p-AMPK? Elucidation of the function of Notch1-LKB1 interactions in Th17 cells may provide interesting insights into Th17 plasticity and uncover targets for therapeutic manipulation.

4.2.3 Regulation of PKCθ expression downstream of IL-6 signaling and hnRNPLL

In Figure 3.2, we show that the expression of *Prkcq* can be induced through IL-6 signaling. Since IL-6 signaling also induced hnRNPLL, we asked if hnRNPLL can regulate *Prkcq* transcript. We saw that hnRNPLL binds to *Prkcq* to a greater degree in Th17 than in iTreg cells, and this was not due to the lower levels of *Prkcq* transcript found in iTreg cells (Figure 2.6). We are confident of this because during our analyses, the RNA immunoprecipitation data were normalized to *Prkcq* expressed in iTregs or in Th17 cells, thereby taking into account any differences in transcript levels between cell types.

These data lead us to further question whether the IL-6-mediated effect on *Prkcq* transcript is solely through its effects on hnRNPLL or is a broader feature of IL-6 signaling, itself. IL-6 signals through JAK kinases to activate STAT3 to modulate gene expression (Schaper and Rose-John, 2015). One means of investigating the hypothesis that IL-6 signaling induces *Prkcq* expression through STAT3 is to perform a chromatin immunoprecipitation (ChIP) assay on the promoter region of *Prkcq* to determine if

STAT3 binding occurs, or is enhanced, following IL-6 treatment. If we do not detect increased STAT3 occupancy on the *Prkcq* promoter, we will need to assess other, less direct means by which IL-6 may influence *Prkcq* expression, such as those exerted by hnRNPLL.

As shown in Figure 3.2, we observed that hnRNPLL can bind *Prkcq* transcript. We can hypothesize that hnRNPLL is necessary for *Prkcq* transcript regulation, as we noted the same pattern of high *Prkcq* expression in Th17 and its low expression in iTreg cells, as measured both by overall transcript levels, and in the amount of transcript bound to hnRNPLL, Figure 3.2. To determine the requirement for hnRNPLL in *Prkcq* transcription and translation, we can block hnRNPLL activity by delivering anti-hnRNPLL into Th17 cells, which have ample PKCθ. If we observe a significant difference in *Prkcq* levels following anti-hnRNPLL treatment, compared to controls, we can conclude that hnRNPLL is necessary for *Prkcq* transcript processing and/or stability. These experiments would allow us to uncouple IL-6 signaling from the actions of hnRNPLL and further determine which signal is more important in *Prkcq* expression. From here, we could further investigate whether hnRNPLL is necessary at the level of *Prkcq* transcriptional or translational processing.

4.2.4 Does LKB1 confer phenotypic stability in Tregs?

A crucial obstacle in Treg therapy is destabilization of the Treg phenotype after transplantation. A key feature of Treg phenotypic stability is the methylation of the TSDR within the *Foxp3* gene locus. Several trials have tried to profile the methylation status of Tregs prior to transplantation to ensure a stable Treg population to the patient. However, even with this precaution, some reports indicate that the Treg therapy is not

entirely efficacious and may even exacerbate inflammation. In Dall'Era et al. (2019), after 12 weeks of Treg therapy, although the percentages of IFNγ-producing CD4 and CD8 T cells decreased, there was an increase in IL-17-producing CD4 and CD8 T cells, compared to baseline. The overall population of Treg cells, characterized as CD4+CD25+ cells, increased slightly. However, the percentage of CD4+CD25+IL-17+ cells also increased, suggesting that the transplanted Tregs may be subject to conversion to IL-17 producing cells, *in vivo* (Dall'Era et al., 2019). These data support a scenario whereby, despite selecting Tregs with a highly demethylated TSDR, these "stable" Tregs cells do not necessarily remain phenotypically stable over time, following transplantation.

LKB1 is a protein of interest regarding Treg stability due to its regulation of fatty acid metabolism, which is crucial to Treg function. It has been shown that LKB1 blocks STAT 4-mediated methylation of the TSDR, to promote stable Tregs. Furthermore, inhibiting LKB1 in the Treg compartment results in systemic autoimmunity, due to a failure of $LKB1^{-/-}$ Tregs to suppress aberrant immune responses (Wu et al. 2017). Additionally, Foxp3 expression is contingent on LKB1 expression, as delivering an shRNA against LKB1 reduced Foxp3 expression, while overexpressing LKB1 increased Foxp3 expression (Su et al., 2019). On the contrary, when LKB1 is knocked out in T cells, effector T cells produce higher levels of cytokines. However, it is not entirely clear whether this phenomenon results from faulty thymocyte development, enhanced effector T cell function, or diminished Treg suppression (MacIver et al., 2011). An intrinsic role for LKB1 in effector cells has not been described. However, data in the

literature that describes a function for LKB1 in driving catabolic metabolism, suggest that LKB1 would act to negatively regulate effector T cell function.

In acute Graft-versus-Host Disease, patient Tregs show reduced functionality and this correlated with low LKB1 expression in the Tregs (Su et al., 2019). In another study, it was determined that LKB1$^{-/-}$ Tregs can secrete inflammatory cytokines, such as IL-17, and when introduced into a host can cause a pro-inflammatory response. This response was related to faulty fatty acid metabolism that occurs in the absence of LKB1 and could be reversed with the addition of fatty acid metabolites (Timilshina et al., 2019). LKB1 is essential to Foxp3 expression, Treg metabolism, and suppressive capacity, making it clear that enhancing LKB1 in Tregs may reasonable therapeutic approach in treating autoimmune disorders.

In this study we provide evidence that implicates LKB1$_S$ as a mediator of Th17-iTreg plasticity. Due to the high correlation between Foxp3 and LKB1, further work needs to be done to determine whether *ex vivo* LKB1 manipulation would produce a population of highly stable Tregs. We demonstrate that downstream of IL-6, a cytokine that induces IL-17 secretion and inhibits Foxp3 expression, PKCθ and hnRNPLL act either separately or in association, to induce LKB1$_S$ expression. Furthermore, we show that when we perturb anabolic metabolism in Th17 cells, we also attenuate LKB1 splicing. This leads to the question of whether blocking LKB1 splicing into its short isoform, either partially or completely, results in more stable Tregs.

To test the effect LKB1$_S$ has on Treg stability we can perform several experiments. First, we can overexpress LKB1$_S$ in Tregs and assess cytokine production. Specifically, is there a correlation between LKB1$_S$ expression and the

production of proinflammatory cytokines? Furthermore, how does LKB1$_S$ overexpression affect TSDR methylation? This can be evaluated using bisulfite sequencing.

If we correlate LKB1$_S$ isoform expression with Treg instability, can we target this pathway to stabilize the Treg phenotype? Our group has previously shown that delivering anti-pPKCθ into CD4 T cells prior to differentiating them into iTregs makes them more potent suppressors, both *in vitro* and *in vivo*. Since we have demonstrated that PKCθ is induced by the proinflammatory cytokine, IL-6, and is involved in the regulating LKB1 splicing, we can hypothesize that inhibiting PKCθ using an antibody delivery strategy may make iTreg cells more resistant to destabilization when challenged with IL-6. To explore this hypothesis, we can repeat the experiment outlined in Figure 3.1, using iTregs that received anti-pPKCθ prior to stimulation. Our readouts for assessing iTreg stability in the presence of IL-6 would be to compare the TSDR demethylation patterns, *Stk11* splice variants, and proinflammatory cytokine production in anti-pPKCθ-treated and untreated iTregs. If anti-pPKCθ treatment increased iTreg stability, we would expect to see protected TSDR demethylation, reduced *Stk11*$_S$ (or relative increases in *Stk11*$_L$), and reduced IL-17 secretion.

We would prefer to perform this experiment first with anti-pPKCθ, as hnRNPL and hnRNPLL are involved in CD45 splicing (Wu et al., 2008; Rothrock et al., 2005). Different isoforms of CD45 are expressed on different types of T cells such as naïve and memory phenotypes providing a means of isolating these cell types in a heterogenous population (Wu et al., 2008). The function of specific CD45 isoforms in Treg biology has not been fully elucidated; however, it has been reported that a population of memory Treg cells that express the CD45RA isoform show a higher

degree of TSDR demethylation at *Foxp3* locus. This population of CD45RA+ Tregs has been documented in patients who have tolerated kidney transplantation well, lending the support of clinical data to this hypothesis (Braza et al., 2014). As such, although anti-hnRNPL or -hnRNPLL may also reduce LKB1 splicing, this inhibition may have off-target effects on CD45 that could produce confounding results.

4.2.5 LKB1$_S$ expression and Th17 function

To our knowledge, our study is the first to identify high expression of LKB1, specifically of LKB1$_S$, in Th17 cells. We demonstrated that, in iTregs, IL-6 upregulates hnRNPLL and increases the levels of *Stk11*$_S$. We would like to further investigate whether *Stk11*$_S$ is necessary and sufficient to establish the Th17 differentiated phenotype. TGF-β and IL-6 synergize to induce RORγt expression of and subsequent IL-17 production (Xiao et al., 2008). In order to test if LKB1$_S$ induction is sufficient to induce cells to adopt a Th17 identity, we can overexpress LKB1$_S$ in naïve T cells and stimulate them with Th17 polarization media. After culturing for 5-7 days, we can use flow cytometry to assay for IL-17 and IL-23 production, as well as for RORγt expression. If there is an increase in cytokine production and/or an increase of RORγt expression, compared to cells not transfected with LKB1$_S$, we can conclude that LKB1$_S$ expression is beneficial to the induction of the Th17 phenotype.

We noted that, when we perturbed glycolysis in Th17 cells, there was a decrease in LKB1$_S$ expression. These Th17 cells still expressed *Rorc,* but also showed increased *Foxp3.* These observations beg the questions: “Is decreased LKB1$_S$ expression due to overall cellular dysfunction?” “Or does inhibiting glycolysis also block *Stk11* splicing?” Another way to ask the latter question is to rephrase it: “Do T cells express LKB1$_S$, as

opposed to $LKB1_L$, to preserve a glycolytic state?" To answer this question, we can isolate RNA from Th17 cells in which LKB1s is overexpressed and ask whether the levels of glycolytic enzymes are enhanced, compared to control cells. Reciprocally, when $LKB1_S$ is introduced into iTreg cells, does this also induce glycolytic enzyme expression? Data that link $LKB1_S$ expression to induction or enhancement of glycolytic enzymes may serve to close an important gap in our understanding of Th17-iTreg plasticity, from the standpoint of metabolic differences.

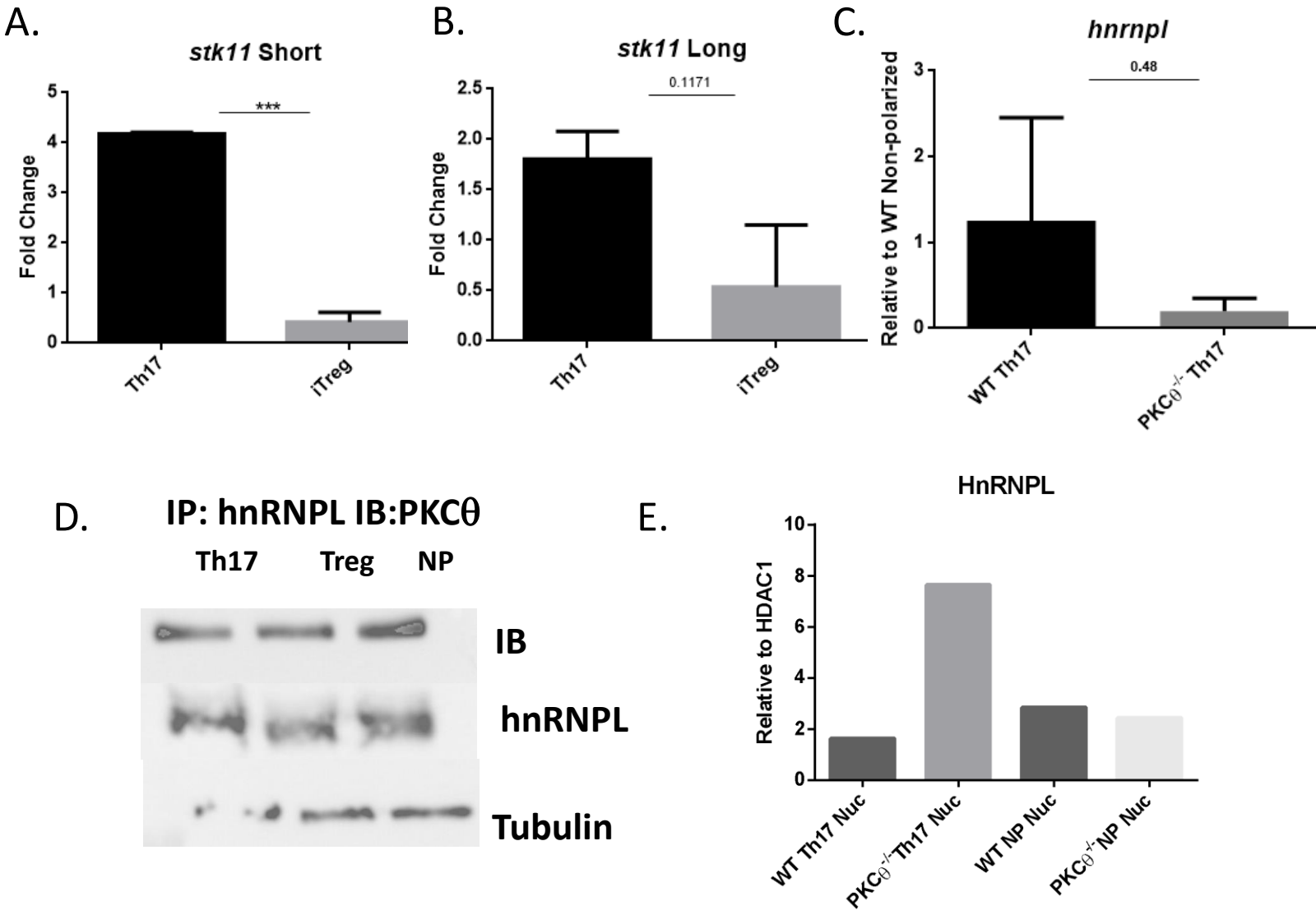

Figure 4.1 HnRNPL regulation of *Stk11* splicing may be regulated by PKCθ

We used RNA-immunoprecipitation to quantify (A) $Stk11_S$ and (B) $Stk11_L$ bound to hnRNPL in Th17 and iTreg cells. WT and PKCθ-/- CD4 T cells were differentiated into NP or Th17 cells. qRT-PCR was used to quantify the expression of (C) *hnrnpl.* Data was analyzed using the ΔΔCt method and values were normalized to input (A-B) and WT NP (C). (D) HnRNPL was immunoprecipitated and immunoblotted for PKCθ to detect binding and with tubulin as a loading control. Data are the mean ± S.E.M of three independent experiments. Unpaired, two-tailed Student's *t*-test was used for analyses; NP $p>0.05$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. (E) Cytoplasmic and nuclear extracts were isolated from WT and PKCθ-/- CD4 T cells were differentiated into NP or Th17 cells and probed for hnRNPL. Data is normalized to HDAC1 for nuclear protein.

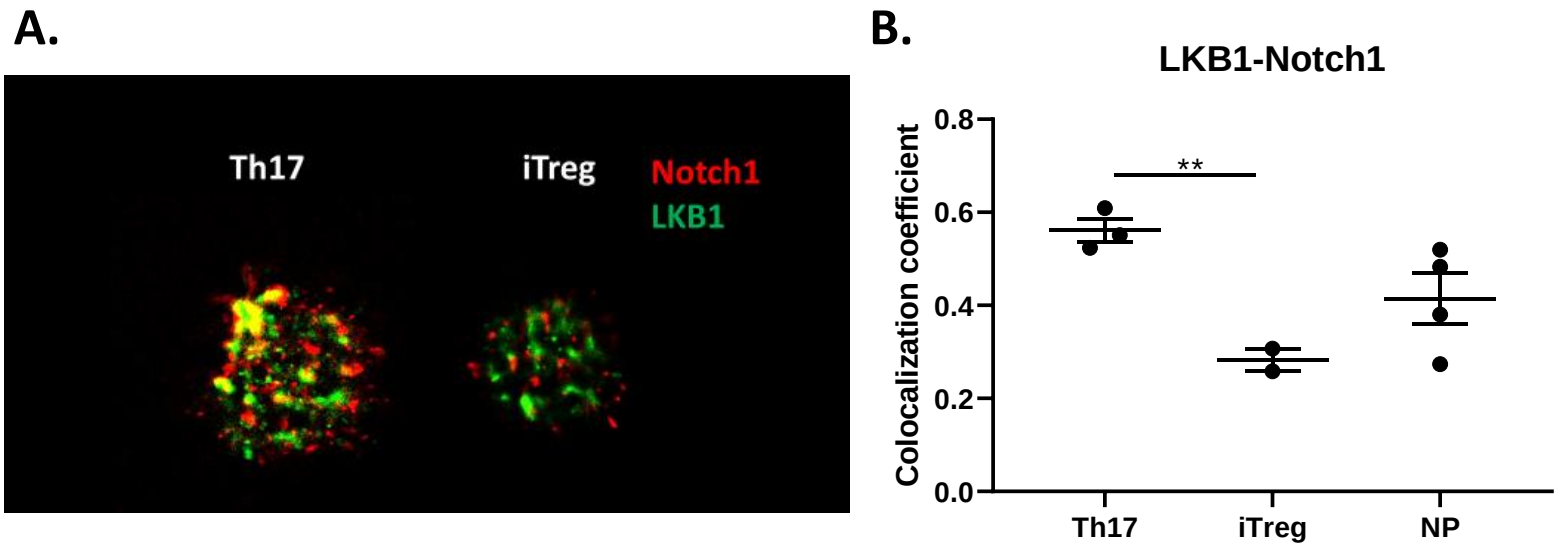

Figure 4.2 LKB1 and Notch1 colocalize in Th17 cells

We STORM to detect differences in LKB1 and Notch1 colocalization. (A) STORM images of Th17 cells and iTregs. Notch1 is in red and LKB1 is in green. (B) The correlation coefficient was determined using a Matlab script from Colorado State University. Data are the mean ± S.E.M of cell images per cell type. Unpaired, two-tailed Student's *t*-test was used for analyses; **p < 0.01.

CHAPTER 5
MATERIALS AND METHODS

5.1 Animals

C57BL/6 mice were purchased from The Jackson Laboratory. PKC$\theta^{-/-}$ mice (C57BL/6 background) were bred in house. All animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Massachusetts, Amherst.

5.2 *In vitro* T cell polarization

CD4 T cells were isolated from spleens using Mojosort (Biolegend). Cells were stimulated on plates coated with anti-hamster IgG (Sigma-Aldrich), anti-CD3e (clone 145-2C11; Biolegend) and anti-CD28 (clone 37.51; BD Biosciences). iTreg cultures were stimulated with these same clones using plate-bound anti-CD3e and soluble anti-CD28. Cells were incubated in a 1:1 mixture of RPMI and DMEM, (Hyclone), supplemented with L-glutamine, sodium pyruvate, pen-strep (GE Life Sciences) and fetal bovine serum (Peak Serum), and 0.34% β-mercaptoethanol. Cells were cultured for 7 days under the following polarizing conditions: Non-polarized (NP): IL-2 (135U/mL; Biolegend); Th17: IL-6 (20ng/mL), TGF-β (5ng/mL; both from Biolegend), anti-IFNγ (10ug/mL, clone XMG 1.2; BioXcell), anti-IL-4 (10ug/mL, clone 41B11; Biolegend and BioXcell); iTreg: IL-2 (135U/mL), TGF-β (20ng/mL), Retinoic Acid (2.5nM, Sigma-Aldrich).

5.3 Flow cytometry

Cells were processed using a Luminex ImageStream^{x} mkII imaging flow cytometer. Cells were fixed and permeabilized with the Foxp3/Transcription Factor Staining Buffer Set (eBioscience). Antibodies used for staining: CD4 FITC (clone H129.19), CD25 APC (clone PC96), Foxp3 PE (clone FJK-16s), Foxp3 PE (clone 150D; all from BD Biosciences), LKB1 (clone D60C5; Cell Signaling Technologies), RORγt PE (clone AFKJS-9; eBioscience), and F(ab')2 IgG QDot625 (Life Technologies). Nuclei were stained using DRAQ5 (ThermoFisher Scientific) Analyses were made using IDEAS, (Luminex Corporation).

5.4 Quantitative Real Time -PCR

RNA was isolated from cells with the Quick-RNA Mini-Prep kit (Zymo Research). cDNA was synthesized with Oligo(dt) 12-18 primer (ThermoFisher Scientific), m-MLV reverse transcriptase and RNasin® plus inhibitor (Promega Corporation), and dNTPs (New England Biolabs). cDNA was used in qRT-PCR reactions with SYBR Green Master Mix (Bimake). Reactions were conducted using a Stratagene Mx3000p (Agilent Technologies). Data were analyzed using the ΔΔCt method. Primer sequences (Integrated DNA Technologies) are listed in Table 5.1.

5.5 Immunoblotting

Cells were lysed in RIPA buffer in the presence of phosphatase and protease inhibitors (Bimake). Protein lysates were resolved on an 8% SDS-PAGE gel and transferred onto a nitrocellulose membrane and blocked with 5% non-fat milk dissolved in 0.2% Tween-20 in PBS. Antibodies used for immunoblotting: hnRNPLL, alpha-tubulin (Cell Signaling

Technologies), Vinculin (ProteinTech), LKB1 (clone Ley37/D6; Santa Cruz Biotechnologies), hnRNPL (clone 4D11; Novus Biologicals. Secondary antibodies used: anti-mouse IgG HRP (GE Amersham) and anti-rabbit IgG (Cell Signaling Technologies and GE Amersham).

5.6 RNA-Immunoprecipitation

Cells were lysed in IP Buffer (Tris-HCL pH 8.0, 200nM NaCl, 0.1% NP-40) in the presence of phosphatase and protease inhibitors (Bimake) and RNasin® plus inhibitor (Promega). Dynabeads protein G® (ThermoFisher Scientific) were coated with anti-hnRNPLL (Cell Signaling Technologies) at room temperature in a solution of 1% BSA (Rocky Mountain Biologicals) in PBS. Lysates and beads were incubated together for 1 hour at 4°C, then washed with IP buffer after incubation. RNA was extracted from the sample and processed for qRT-PCR as described.

5.7 Antibody Delivery

Cells were incubated with the protein transduction mimic synthetic polymer $P_{13}D_5$ at 1μmol/l (Ozay et al., 2016) complexed to 25nmol/l of polyclonal anti-hnRNPLL (Invitrogen, Carlsbad, CA) for 4 hours at 37°C. After incubation, cells were washed in 20U/mL of cold heparin in PBS, then washed in PBS. Cells were resuspended in Th17 polarizing media and differentiated as described.

5.8 RT-PCR

RNA was isolated from cells with the Quick-RNA Mini-Prep kit (Zymo Research). cDNA was synthesized with Oligo(dt) 12-18 primer (ThermoFisher Scientific), m-MLV reverse

transcriptase and RNasin® plus inhibitor (Promega Corporation), and dNTPs (New England Biolabs). RT-PCR reactions were performed with Phusion polymerase (New England Biolabs). The reactions were run on a Mastercycler Thermal Cycler (Eppendorf). The samples were resolved on a 2% agarose gel.

5.9 Dichloroacetate (DCA) Treatment

Th17 cells were differentiated in Th17 polarization media for 5 days. On day 5, 10mM of DCA (Gerriets et al., 2015) was added to the culture and cells were harvested 48 hours later.

5.10 Immunoprecipitation

Cells were lysed in IP Buffer (Tris-HCL pH 8.0, 200nM NaCl, 0.1% NP-40) in the presence of phosphatase and protease inhibitors (Bimake) and RNasin® plus inhibitor (Promega). Dynabeads protein G® (ThermoFisher Scientific) were coated with anti-hnRNPLL (Cell Signaling Technologies) at room temperature in a solution of 1% BSA (Rocky Mountain Biologicals) in PBS. Lysates and beads were incubated together for 1 hour at 4°C, then washed with IP buffer after incubation. Protein lysates were resolved on an 8% SDS-PAGE gel and transferred onto a nitrocellulose membrane and blocked with 5% non-fat milk dissolved in 0.2% Tween-20 in PBS.

5.11 Cytoplasmic and Nuclear Extract Isolation

Protein lysates were extracted using the NER-PER™ Nuclear and Cytoplasmic Extraction Reagents (ThermoFisher Scientific). These lysates were then used according to the immunoblot protocol.

5.12 STORM Microscopy

Cells were placed on poly-d-lysine coated plate (MatTeK) for 1 hour at room temperature. Cells were fixed and permeabilized using the Foxp3/Transcription Factor Staining Buffer Set (eBioscience). Cells were stained with LKB1 Ley37D/G6 AF488 and Notch1 mN1a AF647 (both from Santa Cruz Biotechnologies). Images were analyzed with NIS elements and Matlab.

5.13 Statistics

Data are the mean ± SEM; all experiments were repeated at least three times. Unpaired, two-tailed Student's *t*-test and two-way ANOVA with post-Bonferroni test were applied for statistical comparison using GraphPad Prism 8 software. p values of ≤ 0.05 were considered significant.

Table 5.1 Antibodies used in this study

Target	Clone	Company
hamster IgG	Polyclonal	Sigma-Aldrich
CD3e	145-2C11	Biolegend
CD28	37.51	BD Biosciences
IFNg	XMG 1.2	BioXcell
IL-4	41B11	Biolegend
IL-4	41B11	BioXcell
CD4	H129.19	BD Biosciences
CD25	PC96	BD Biosciences
Foxp3	FJK-16s	BD Biosciences
Foxp3	150D	BD Biosciences
LKB1	D60C5	Cell Signaling Technologies
LKB1	Ley37D/G6	Santa Cruz Biotechnology
RORgT	AFKJS-9	eBioscience
F(ab')2 IgG		Life Technologies
hnRNPLL	Polyclonal	Cell Signaling Technologies
hnRNPLL	Polyclonal	Invitrogen
a-tubulin	Polyclonal	Cell Signaling Technologies
mouse IgG HRP	Polyclonal	GE Amersham
rabbit IgG HRP	Polyclonal	GE Amersham
rabbit IgG HRP	Polyclonal	Cell Signaling Technologies
HnRNPL	4D11	Novus Biologicals

Table 5.2 Primers used in this study

Primer Name	Forward Sequence	Reverse Sequence
Stk11 Common	GACTCCGAGACCTTATGCCG	TTCTTGACGTTGGCCTCTCC
Stk11$_L$	CATTATCTACACCCAGGACTTCACA	CGCATGCATCCTCGCTAA
Stk11$_S$	CCTGCAAGCAGCAGTGAC	CCAACGTCCCGAAGTGAG
Hnrnpll	GTGACGAGTACGCTGTGGAA	CTGGGAACAACTGAGTGCTG
Il17f	GAGGATAACACTGTGAGAGTTGAC	GAGTTCATGGTGCTGTCTTCC
Prkcq	ATGGACAACCCCTTCTACCC	GCGGATGTCTCCTCTCACTC
Rorc	CACGGCCCTGGTTCTCAT	GCAGATGTTCCACTCTCCTCTTCT
Foxp3	CTTATCCGATGGGCCATCCTGGAAG	TTCCAGGTGGCGGGGTGGTTTCTG
ActB	GGCTGTATTCCCCTCCATCG	CCAGTTGGTAACAATGCCATGT
Ptprc	GGCAACACCTACACCCA	GCTTGCAGGCCCAGA

BIBLIOGRAPHY

Almeida, L., Lochner, M., Berod, L., & Sparwasser, T. (2016). Metabolic pathways in T cell activation and lineage differentiation. In *Seminars in Immunology*, 28(5):514–524. https://doi.org/10.1016/j.smim.2016.10.009

Angelin, A., Gil-de-Gómez, L., Dahiya, S., Jiao, J., Guo, L., Levine, M. H., Wang, Z., Quinn, W. J., Kopinski, P. K., Wang, L., Akimova, T., Liu, Y., Bhatti, T. R., Han, R., Laskin, B. L., Baur, J. A., Blair, I. A., Wallace, D. C., Hancock, W. W., & Beier, U. H. (2017). Foxp3 Reprograms T Cell Metabolism to Function in Low-Glucose, High-Lactate Environments. *Cell Metabolism*, 25:1282–1293. https://doi.org/10.1016/j.cmet.2016.12.018

Bentley, D. L. (2014). Coupling mRNA processing with transcription in time and space. In *Nature Reviews Genetics*, 15(3):163–175. https://doi.org/10.1038/nrg3662

Berg-Brown, N. N., Gronski, M. A., Jones, R. G., Elford, A. R., Deenick, E. K., Odermatt, B., Littman, D. R., & Ohashi, P. S. (2004). PKCθ Signals Activation versus Tolerance In Vivo. *Journal of Experimental Medicine*, 199(6):743–752. https://doi.org/10.1084/jem.20031022

Berod, L., Friedrich, C., Nandan, A., Freitag, J., Hagemann, S., Harmrolfs, K., Sandouk, A., Hesse, C., Castro, C. N., BäHre, H., Tschirner, S. K., Gorinski, N., Gohmert, M., Mayer, C. T., Huehn, J., Ponimaskin, E., Abraham, W. R., MüLler, R., Lochner, M., & Sparwasser, T. (2014). De novo fatty acid synthesis controls the fate between regulatory T and T helper 17 cells. *Nature Medicine*, 20(11):1327–1333. https://doi.org/10.1038/nm.3704

Blagih, J., Krawczyk, C. M., & Jones, R. G. (2012). LKB1 and AMPK: central regulators of lymphocyte metabolism and function. *Immunological Reviews*, 249(1), 59–71. https://doi.org/10.1111/j.1600-065X.2012.01157.x

Bluestone, J. A., Buckner, J. H., Fitch, M., Gitelman, S. E., Gupta, S., Hellerstein, M. K., Herold, K. C., Lares, A., Lee, M. R., Li, K., Liu, W., Long, S. A., Masiello, L. M., Nguyen, V., Putnam, A. L., Rieck, M., Sayre, P. H., & Tang, Q. (2015). Type 1 diabetes immunotherapy using polyclonal regulatory T cells. *Science Translational Medicine*, 7(315):189. http://doi.org/10.1126/scitranslmed.aad4134

Bluestone, J. A., & Tang, Q. (2018). Treg cells-the next frontier of cell therapy: Will regulatory T cells be a frontline therapy for autoimmunity and other diseases? *Science*, 362(6411):154–155. https://doi.org/10.1126/science.aau2688

Boonpiyathad, T., Satitsuksanoa, P., Akdis, M., & Akdis, C. A. (2019). Il-10 producing T and B cells in allergy. *Seminars in Immunology*, 44:101326 https://doi.org/10.1016/j.smim.2019.101326

Butte, M. J., Lee, S. J., Jesneck, J., Keir, M. E., & Haining, W. N. (2012). CD28 Costimulation Regulates Genome-Wide Effects on Alternative Splicing. *PLoS ONE*, 7(6):40032. https://doi.org/10.1371/journal.pone.0040032

Chapman, N. M., Boothby, M. R., & Chi, H. (2019). Metabolic coordination of T cell quiescence and activation. *Nature Reviews Immunology*, 20:55-70. https://doi.org/10.1038/s41577-019-0203-y

Chavele, K.-M., & Ehrenstein, M. R. (2011). Regulatory T-cells in systemic lupus erythematosus and rheumatoid arthritis. *FEBS Letters*, 585(23):3603–3610. https://doi.org/10.1016/j.febslet.2011.07.043

Chen X, Das R, Komorowski R, Beres A, Hessner MJ, Mihara M, Drobyski WR (2009) Blockade of interleukin-6 signaling augments regulatory T-cell reconstitution and attenuates the severity of graft-versus-host disease. *Blood*, 114:891-900. https://doi.org/10.1182/blood-2009-01-197178

Chen, S., Fang, L., Guo, W., Zhou, Y., Yu, G., Li, W., Dong, K., Liu, J., Luo, Y., Wang, B., Li, Z., Zhao, C., Sun, Z., Shen, Y., Leng, Q., Zhou, D., Han, Z., Huang, H., Ren, H., … Feng, X. (2018). Control of T reg cell homeostasis and immune equilibrium by Lkb1 in dendritic cells. *Nature Communications*, *9*(1). https://doi.org/10.1038/s41467-018-07545-8

Dahmani, R., Just, P. A., Delay, A., Canal, F., Finzi, L., Prip-Buus, C., Lambert, M., Sujobert, P., Buchet-Poyau, K., Miller, E., Cavard, C., Marmier, S., Terris, B., Billaud, M., & Perret, C. (2015). A novel LKB1 isoform enhances AMPK metabolic activity and displays oncogenic properties. *Oncogene*, 34(18):2337–2346. https://doi.org/10.1038/onc.2014.182

Dall'Era, M., Pauli, M. L., Remedios, K., Taravati, K., Sandova, P. M., Putnam, A. L., Lares, A., Haemel, A., Tang, Q., Hellerstein, M., Fitch, M., McNamara, J., Welch, B., Bluestone, J. A., Wofsy, D., & Rosenblum, M. D. (2019). Adoptive Treg Cell Therapy in a Patient With Systemic Lupus Erythematosus. *Arthritis & Rheumatology*, 71(3):431–440. https://doi.org/10.1002/art.40737

Deng, G., Song, X., Fujimoto, S., Piccirillo, C. A., Nagai, Y., & Greene, M. I. (2019). Foxp3 post-translational modifications and treg suppressive activity. In *Frontiers in Immunology*, 10:2486. https://doi.org/10.3389/fimmu.2019.02486

Dodington, D. W., Desai, H. R., & Woo, M. (2018). JAK/STAT – Emerging Players in Metabolism. *Trends in Endocrinology and Metabolism*, 29(1):55–65. https://doi.org/10.1016/j.tem.2017.11.001

Duhen, T., Duhen, R., Lanzavecchia, A., Sallusto, F., & Campbell, D. J. (2012). Functionally distinct subsets of human FOXP3 + Treg cells that phenotypically mirror effector Th cells. *Blood*, 119(19), 4430–4440. https://doi.org/10.1182/blood-2011-11-392324

Ferrara J.L.M & Levine J.E. (2008). A new approach to therapy for acute GvHD. *Cellular Therapy and Transplantation*, 1(1). http://doi.org/10.3205/ctt2008-05-26-002-en

Ferreira, L. M. R., Muller, Y. D., Bluestone, J. A., & Tang, Q. (2019). Next-generation regulatory T cell therapy. *Nature Reviews Drug Discovery*, 18(10):749–769. https://doi.org/10.1038/s41573-019-0041-4

Fisher SA, Lamikanra A, Doree C, Gration B, Tsang P, Danby RD, Roberts DJ (2016) Increased regulatory T cell graft content is associated with improved outcome in hematopoietic stem cell transplantation: a systematic review. *British Journal of Hematology*, 176:448-463. https://doi.org/10.1111/bjh.14433

Fox, C. J., Hammerman, P. S., & Thompson, C. B. (2005). Fuel feeds function: Energy metabolism and the T-cell response. In *Nature Reviews Immunology* (Vol. 5, Issue 11, pp. 844–852). Nature Publishing Group. https://doi.org/10.1038/nri1710

Galgani, M., De Rosa, V., & Matarese, G. (2015). T cell metabolism and susceptibility to autoimmune diseases. *Molecular Immunology*, 68(2), 558–563. https://doi.org/10.1016/j.molimm.2015.07.035

Gerriets, V. A., Kishton, R. J., Nichols, A. G., MacIntyre, A. N., Inoue, M., Ilkayeva, O., Winter, P. S., Liu, X., Priyadharshini, B., Slawinska, M. E., Haeberli, L., Huck, C., Turka, L. A.,Wood, K. C., Hale, L. P., Smith, P. A., Schneider, M. A., MacIver, N. J., Locasale, J. W., … Rathmell, J. C. (2015). Metabolic programming and PDHK1 control CD4+ T cell subsets and inflammation. *Journal of Clinical Investigation*, 125(1), 194–207. https://doi.org/10.1172/JCI76012

Geuens, T., Bouhy, D., & Timmerman, V. (2016). The hnRNP family: insights into their role in health and disease. In *Human Genetics*, 135(8):851–867. https://doi.org/10.1007/s00439-016-1683-5

Gianchecchi, E., & Fierabracci, A. (2018). Inhibitory receptors and pathways of lymphocytes: The role of PD-1 in Treg development and their involvement in autoimmunity onset and cancer progression. *Frontiers in Immunology*, 9:2374. https://doi.org/10.3389/fimmu.2018.02374

Gualdoni, G. A., Mayer, K. A., Göschl, L., Boucheron, N., Ellmeier, W., & Zlabinger, G. J. (2016). The AMP analog AICAR modulates the Treg/Th17 axis through enhancement of fatty acid oxidation. *FASEB Journal*, 30(11):3800–3809. https://doi.org/10.1096/fj.201600522R

He N, Fan W, Henriquez B, Yu RT, Atkins AR, Liddle C, Zheng Y, Downes M, Evans RM (2017) Metabolic control of regulatory T cell (Treg) survival and function by LKB1. *PNAS*. 114:12542-12547. http://doi.org/10.1073/pnas.1715363114

Hemminki, A., Markie, D., Tomlinson, I., Avizienyte, E., Roth, S., Loukola, A., Bignell, G., Warren, W., Aminoff, M., Höglund, P., Järvinen, H., Kristo, P., Pelin, K., Ridanpää, M., Salovaara, R., Toro, T., Bodmer, W., Olschwang, S., Olsen, A. S., Stratton, M. R., de la Chapelle, A., and Aal- tonen, L. A. (1998) A serine/threonine kinase gene defective in Peutz- Jeghers Syndrome. *Nature*, 391:184-187.

Hill L, Alousi A, Kebriaei P, Mehta R, Rezvani K, Shpall E (2018) New and emerging therapies for acute and chronic graft *versus* host disease. *Therapeutic Advances in Hematology*. 9:21-46. http://doi.org/10.1177/2040620717741860

Hoefig, K. P., & Heissmeyer, V. (2018). Posttranscriptional regulation of T helper cell fate decisions. *Journal of Cell Biology*, 217(8):2615–2631. https://doi.org/10.1083/jcb.201708075

Holmes, N. (2006). CD45: All is not yet crystal clear. *Immunology*, 117(2):145–155. https://doi.org/10.1111/j.1365-2567.2005.02265.x

Horwitz, D. A., Fahmy, T. M., Piccirillo, C. A., & Cava, A. La. (2019). Feature Review Rebalancing Immune Homeostasis to Treat Autoimmune Diseases. *Trends in Immunology*, 40:888–908. https://doi.org/10.1016/j.it.2019.08.003

House, A. E., & Lynch, K. W. (2008). Regulation of alternative splicing: More than just the ABCs. In *Journal of Biological Chemistry*, 283(3):1217–1221. https://doi.org/10.1074/jbc.R700031200

Howie, D., Cobbold, S. P., Adams, E., Ten Bokum, A., Necula, A. S., Zhang, W., Huang, H., Roberts, D. J., Thomas, B., Hester, S. S., Vaux, D. J., Betz, A. G., & Waldmann, H. (2017). Foxp3 drives oxidative phosphorylation and protection from lipotoxicity. *JCI Insight*, 2(3): e89160. https://doi.org/10.1172/jci.insight.89160

Isakov N, Altman A. Protein kinase C θ in T cell activation. (2002) *Annu Rev Immunol.* 20(1):761–94. http://doi.org/:10.1146/annurev.immunol.20.100301.064807

Ivanov, I. I., Mckenzie, B. S., Zhou, L., Tadokoro, C. E., Lepelley, A., Lafaille, J. J., Cua, D. J., & Littman, D. R. (2006). The Orphan Nuclear Receptor RORγt Directs the Differentiation Program of Proinflammatory IL-17 + T Helper Cells. *Cell*, 126:1121-1133. https://doi.org/10.1016/j.cell.2006.07.035

Iwakura, Y., Ishigame, H., Saijo, S., & Nakae, S. (2011). Functional Specialization of Interleukin-17 Family Members. *Immunity*, 34(2):149–162. https://doi.org/10.1016/j.immuni.2011.02.012

Joller, N., Lozano, E., Burkett, P. R., Patel, B., Xiao, S., Zhu, C., Xia, J., Tan, T. G., Sefik, E., Yajnik, V., Sharpe, A. H., Quintana, F. J., Mathis, D., Benoist, C., Hafler, D. A., & Kuchroo, V. K. (2014). Treg cells expressing the coinhibitory molecule TIGIT selectively

inhibit proinflammatory Th1 and Th17 cell responses. *Immunity*, 40: 569-581. https://doi.org/10.1016/j.immuni.2014.02.012

Kanamori, M., Nakatsukasa, H., Okada, M., Lu, Q., & Yoshimura, A. (2016). Induced Regulatory T Cells: Their Development, Stability, and Applications. *Trends in Immunology*, 37(11):803-811. https://doi.org/10.1016/j.it.2016.08.012

Kempkes, R. W. M., Joosten, I., Koenen, H. J. P. M., & He, X. (2019). Metabolic Pathways Involved in Regulatory T Cell Functionality. *Frontiers in Immunology*, 10:2839. https://doi.org/10.3389/fimmu.2019.02839

Kitagawa, Y., & Sakaguchi, S. (2017). Molecular control of regulatory T cell development and function. *Current Opinion in Immunology*, 49:64-70. https://doi.org/10.1016/j.coi.2017.10.002

Kurtulus, S., Sakuishi, K., Ngiow, S. F., Joller, N., Tan, D. J., Teng, M. W. L., Smyth, M. J., Kuchroo, V. K., & Anderson, A. C. (2015). TIGIT predominantly regulates the immune response via regulatory T cells. *Journal of Clinical Investigation*, 125(11), 4053–4062. https://doi.org/10.1172/JCI81187

Kwon, M.-J., Ma, J., Ding, Y., Wang, R., & Sun, Z. (2012). Protein Kinase C-θ Promotes Th17 Differentiation via Upregulation of Stat3. *The Journal of Immunology*, 188(12):5887–5897. https://doi.org/10.4049/jimmunol.1102941

Leonard, W. J., & O'shea, J. J. (1998). JAKS AND STATS: Biological Implications *. *Annu. Rev. Immunol*, 16:293-322.

Le Texier, L., Lineburg, K. E., Cao, B., McDonald-Hyman, C., Leveque-El Mouttie, L., Nicholls, J., Melino, M., Nalkurthi, B. C., Alexander, K. A., Teal, B., Blake, S. J., Souza-Fonseca-Guimaraes, F., Engwerda, C. R., Kuns, R. D., Lane, S. W., Teng, M., Teh, C., Gray, D., Clouston, A. D., … MacDonald, K. P. (2016). Autophagy-dependent regulatory T cells are critical for the control of graft-versus-host disease. *JCI Insight*, 1(15), 86850. https://doi.org/10.1172/jci.insight.86850

Lochner, M., Berod, L., & Sparwasser, T. (2015). Fatty acid metabolism in the regulation of T cell function. *Trends in Immunology*, 36(2):81–91. https://doi.org/10.1016/j.it.2014.12.005

Lucas, P. C., McAllister-Lucas, L. M., & Nunez, G. (2004). NF-κB in signaling in lymphocytes: A new cast of characters. *Journal of Cell Science*, 117(1):31–39. https://doi.org/10.1242/jcs.00904

Lynch, K. W. (2004). Consequences of regulated pre-mRNA splicing in the immune system. In *Nature Reviews Immunology*, 4(12):931–940. https://doi.org/10.1038/nri1497

Ma, J., Ding, Y., Fang, X., Wang, R., & Sun, Z. (2012). Protein Kinase C-θ Inhibits Inducible Regulatory T Cell Differentiation via an AKT-Foxo1/3a–Dependent Pathway. *The Journal of Immunology*, *188*(11):5337–5347. https://doi.org/10.4049/jimmunol.1102979

Ma, E. H., Poffenberger, M. C., Wong, A. H. T., & Jones, R. G. (2017). The role of AMPK in T cell metabolism and function. *Current Opinion in Immunology*, 46: 45–52. https://doi.org/10.1016/j.coi.2017.04.004

MacIver NJ, Blagih J, Saucillo DC, Tonelli L, Griss T, Rathmell JC, Jones RG (2011) The Liver Kinase B1 Is a Central Regulator of T Cell Development, Activation, and Metabolism. *J Immunol*, 187:4187-4198. https://doi.org/10.4049/jimmunol.1100367

McCuaig, R. D., Dunn, J., Li, J., Masch, A., Knaute, T., Schutkowski, M., Zerweck, J., & Rao, S. (2015). PKC-theta is a novel SC35 splicing factor regulator in response to T cell activation. *Frontiers in Immunology*, 6:562. https://doi.org/10.3389/fimmu.2015.00562

McGeachy, M. J., & Cua, D. J. (2008). Th17 Cell Differentiation: The Long and Winding Road. *Immunity*, 28 (4) 445–453. https://doi.org/10.1016/j.immuni.2008.03.001

Morishima, N., Mizoguchi, I., Takeda, K., Mizuguchi, J., & Yoshimoto, T. (2009). TGF-β is necessary for induction of IL-23R and Th17 differentiation by IL-6 and IL-23. *Biochemical and Biophysical Research Communications*, 386(1):105–110. https://doi.org/10.1016/j.bbrc.2009.05.140

Moutuou MM, Pagé G, Zaid I, Lesage S and Guimond M (2018) Restoring T Cell Homeostasis After Allogeneic Stem Cell Transplantation; Principal Limitations and Future Challenges. *Front. Immunol.* 9:1237. http://doi.org/10.3389/fimmu.2018.01237

Mucida, D., Park, Y., Kim, G., Turovskaya, O., Scott, I., Kronenberg, M., & Cheroutre, H. (2007). Reciprocal TH17 and regulatory T cell differentiation mediated by retinoic acid. *Science*, 317(5835):256–260. https://doi.org/10.1126/science.1145697

Nassereddine S, Rafei H, Elbahesh E, Tabbara I (2017) Acute Graft *versus* Host Disease: A Comprehensive Review. *Anticancer Research.* 37:1547-1555. http://doi.org/10.21873/anticanres.11483

Nish, S. A., Schenten, D., Wunderlich, T., Pope, S. D., Gao, Y., Hoshi, N., Yu, S., Yan, X., Lee, H. K., Pasman, L., Brodsky, I., Yordy, B., Zhao, H., Bruning, J., & Medzhitov, R. (2014). T cell-intrinsic role of IL-6 signaling in primary and memory responses. *ELife*, 2014(3):e01949. https://doi.org/10.7554/eLife.01949

Oberdoerffer, S., Moita, L. F., Neems, D., Freitas, R. P., Hacohen, N., & Rao, A. (2008). Regulation of CD45 alternative splicing by heterogeneous ribonucleoprotein, hnRNPLL. *Science*, 321(5889):686–691. https://doi.org/10.1126/science.1157610

Ohkura, N., Hamaguchi, M., Morikawa, H., Sugimura, K., Tanaka, A., Ito, Y., Osaki, M., Tanaka, Y., Yamashita, R., Nakano, N., Huehn, J., Fehling, H. J., Sparwasser, T., Nakai, K., & Sakaguchi, S. (2012). T Cell Receptor Stimulation-Induced Epigenetic Changes and Foxp3 Expression Are Independent and Complementary Events Required for Treg Cell Development. *Immunity*, *37*(5), 785–799. https://doi.org/10.1016/j.immuni.2012.09.010

Osborne, B. A., & Minter, L. M. (2007). Notch signalling during peripheral T-cell activation and differentiation. *Nature Reviews Immunology*, 7(1):64–75. https://doi.org/10.1038/nri1998

O'Shea, J. J., & Plenge, R. (2012). JAK and STAT Signaling Molecules in Immunoregulation and Immune-Mediated Disease. *Immunity*, 36:542-550. https://doi.org/10.1016/j.immuni.2012.03.014

Owen, Punt, & Stranford. (2013). Kuby Immunology 7th edition.

Owen, D. L., Sjaastad, L. E., & Farrar, M. A. (2019). Regulatory T Cell Development in the Thymus. *The Journal of Immunology*, *203*(8), 2031–2041. https://doi.org/10.4049/jimmunol.1900662

Ozay, E. I., Gonzalez-Perez, G., Torres, J. A., Vijayaraghavan, J., Lawlor, R., Sherman, H. L., Garrigan, D. T., Burnside, A. S., Osborne, B. A., Tew, G. N., & Minter, L. M. (2016). Intracellular delivery of anti-pPKCθ (Thr538) via protein transduction domain mimics for immunomodulation. *Molecular Therapy*, 24(12):2118–2130. https://doi.org/10.1038/mt.2016.177

Ozay, E. I., Sherman, H. L., Mello, V., Trombley, G., Lerman, A., Tew, G. N., Yadava, N., & Minter, L. M. (2018). Rotenone treatment reveals a role for electron transport complex I in the subcellular localization of key transcriptional regulators during T helper cell differentiation. *Frontiers in Immunology*, 9:1284. https://doi.org/10.3389/fimmu.2018.01284

Ozay, E.I., Shanthalingam S., Torres, J.A., Osborne B.A., Tew G.N., & Minter L.M. Protein Kinase C theta modulates PCMT1 through hnRNPL to regulate FOXP3 stability in regulatory T cells. *Molecular Therapy*, submitted.

Paz I, Kosti I, Ares M Jr, Cline M, Mandel-Gutfreund Y. (2014) RBPmap: a web server for mapping binding sites of RNA-binding proteins. *Nucleic Acids Res*., 2014

Preußner, M., Schreiner, S., Hung, L.-H., Porstner, M., Jä Ck, H.-M., Benes, V., Rä Tsch, G., & Bindereif, A. (2012). *H*nRNP L and L-like cooperate in multiple-exon regulation of CD45 alternative splicing. *Nucleic Acids Research*, 40:12. https://doi.org/10.1093/nar/gks221

Priyaharshini M., Kotlo K.U., Dudeja P.K., & Layden B.T. (2018). Role of Short Chain Fatty Acid Receptors in Intestinal Physiology and Pathophysiology. *Compr Physiol.*, 8(3):1091-1115. http://doi.org/10.1002/cphy.c170050

Raffin, C., Vo, L. T., & Bluestone, J. A. (2019). Treg cell-based therapies: challenges and perspectives. *Nature Reviews Immunology*. https://doi.org/10.1038/s41577-019-0232-6

Ramlal R, Hildebrandt GC (2017) Advances in the Use of Regulatory T-Cells for the Prevention and Therapy of Graft versus Host Disease. *Biomedicines*, 5:23. https://doi.org/10.3390/biomedicines5020023

Ren, J., & Li, B. (2017). The Functional Stability of FOXP3 and RORγt in Treg and Th17 and Their Therapeutic Applications. *Advances in Protein Chemistry and Structural Biology*, 107:155–189. https://doi.org/10.1016/bs.apcsb.2016.10.002

Rothstein, D. M., & Camirand, G. (2015). New insights into the mechanisms of Treg function. *Curr Opin Organ Transplant*. 20(4): 376–384. https://doi.org/10.1097/MOT.0000000000000212

Samanta, A., Li, B., Song, X., Bembas, K., Zhang, G., Katsumata, M., Saouaf, S. J., Wang, Q., Hancock, W. W., Shen, Y., & Greene, M. I. (2008). TGF-β and IL-6 signals modulate chromatin binding and promoter occupancy by acetylated FOXP3. *PNAS*, 105(37):14023–14027. https://doi.org/10.1073/pnas.0806726105

Schaper, F., & Rose-John, S. (2015). Interleukin-6: Biology, signaling and strategies of blockade. *Cytokine and Growth Factor Reviews*, 26(5):475–487. https://doi.org/10.1016/j.cytogfr.2015.07.004

Scheinecker, C., Göschl, L., & Bonelli, M. (2019). Treg cells in health and autoimmune diseases: New insights from single cell analysis. *Journal of Autoimmunity*. https://doi.org/10.1016/j.jaut.2019.102376

Schlöder J, Berges C, Tuettenberg A and Jonuleit H (2017) Novel Concept of CD4-Mediated Activation of Regulatory T Cells for the Treatment of Graft-Versus-Host Disease. *Front. Immunol*. 8:1495. http://doi.org/10.3389/fimmu.2017.01495

Schmitt, N., & Ueno, H. (2015). Regulation of human helper T cell subset differentiation by cytokines. *Current Opinion in Immunology*, 34:130-136. https://doi.org/10.1016/j.coi.2015.03.007

Sen, S., Wang, F., Zhang, J., He, Z., Ma, J., Gwack, Y., Xu, J., & Sun, Z. (2018). SRC1 promotes Th17 differentiation by overriding Foxp3 suppression to stimulate RORγt activity in a PKC-θ-dependent manner. *PNAS*, 115(3):458–467. https://doi.org/10.1073/pnas.1717789115

Sharif, K., Watad, A., Bragazzi, N. L., Adawi, M., Amital, H., & Shoenfeld, Y. (2017). Coffee and autoimmunity: More than a mere hot beverage! *Autoimmunity Reviews*, 16(7):712–721. https://doi.org/10.1016/j.autrev.2017.05.007

Shenasa, H., & Hertel, K. J. (2019). Combinatorial regulation of alternative splicing. In *Biochimica et Biophysica Acta - Gene Regulatory Mechanisms*, 1862: 11–12. https://doi.org/10.1016/j.bbagrm.2019.06.003

Shevach, E. M. (2009). Mechanisms of Foxp3+ T Regulatory Cell-Mediated Suppression. *Immunity*. 30:636-645. https://doi.org/10.1016/j.immuni.2009.04.010

Shi, H., & Chi, H. (2019). Metabolic Control of Treg Cell Stability, Plasticity, and Tissue-Specific Heterogeneity. *Frontiers in Immunology*, 10:2716. https://doi.org/10.3389/fimmu.2019.02716

Shono, Y., & Van Den Brink, M. R. M. (2018). Gut microbiota injury in allogeneic haematopoietic stem cell transplantation. *Nature Reviews Cancer*, 18(5): 283–295. https://doi.org/10.1038/nrc.2018.10

Smith M, Zakrzewski J, James S, Sadelain M (2018) Posttransplant chimeric antigen receptor therapy, *Blood*. 131:1045-1052. https://doi.org/10.1182/blood-2017-08-752121

Su, X., Wang, Q., Guo, W., Pei, X., Niu, Q., Liu, M., Liu, Y., Chen, S., Feng, S., & He, Y. (2019). Loss of Lkb1 impairs Treg function and stability to aggravate graft-versus-host disease after bone marrow transplantation. *Cellular & Molecular Immunology*, September: 1–13. https://doi.org/10.1038/s41423-019-0312-3

Sun, L., Fu, J., & Zhou, Y. (2017). Metabolism controls the balance of Th17/T-regulatory cells. *Frontiers in Immunology*, 8:1632. https://doi.org/10.3389/fimmu.2017.01632

Sutcliffe, E. L., & Rao, S. (2011). Duplicity of protein kinase C-θ. *Transcription*, *2*(4):189–192. https://doi.org/10.4161/trns.2.4.16565

Sutcliffe, E. L., Bunting, K. L., He, Y. Q., Li, J., Phetsouphanh, C., Seddiki, N., Zafar, A., Hindmarsh, E. J., Parish, C. R., Kelleher, A. D., McInnes, R. L., Taya, T., Milburn, P. J., & Rao, S. (2011). Chromatin-Associated Protein Kinase C-θ Regulates an Inducible Gene Expression Program and MicroRNAs in Human T Lymphocytes. *Molecular Cell*, l 41:704-719. https://doi.org/10.1016/j.molcel.2011.02.030

Sutcliffe, E. L., Bunting, K. L., He, Y. Q., Li, J., Phetsouphanh, C., Seddiki, N., Zafar, A., Hindmarsh, E. J., Parish, C. R., Kelleher, A. D., McInnes, R. L., Taya, T., Milburn, P. J., & Rao, S. (2011). Chromatin-Associated Protein Kinase C-θ Regulates an Inducible Gene Expression Program and MicroRNAs in Human T Lymphocytes. *Molecular Cell*, 41(6):704–719. https://doi.org/10.1016/j.molcel.2011.02.030

Tamás, P., Macintyre, A., Finlay, D., Clarke, R., Feijoo-Carnero, C., Ashworth, A., & Cantrell, D. (2010). LKB1 is essential for the proliferation of T-cell progenitors and mature peripheral T cells. *European Journal of Immunology*, 40(1):242–253. https://doi.org/10.1002/eji.200939677

Tanaka, T., Narazaki, M., & Kishimoto, T. (2014). Il-6 in inflammation, Immunity, And disease. *Cold Spring Harbor Perspectives in Biology*, 6:a016295. https://doi.org/10.1101/cshperspect.a016295

Thibert, C., Perret, C., & Billaud, M. (2015). AMPK potentiation by LKB1 isoforms. *Oncotarget*, 6(34):35139–35140. https://doi.org/10.18632/oncotarget.6127

Timilshina, M., You, Z., Lacher, S. M., Acharya, S., Jiang, L., Kang, Y., Kim, J. A., Chang, H. W., Kim, K. J., Park, B., Song, J. H., Ko, H. J., Park, Y. Y., Ma, M. J., Nepal, M. R., Jeong, T. C., Chung, Y., Waisman, A., & Chang, J. H. (2019). Activation of Mevalonate Pathway via LKB1 Is Essential for Stability of Treg Cells. *Cell Reports*, 27(10):2948-2961. https://doi.org/10.1016/j.celrep.2019.05.020

Trzonkowski, P., Szaryńska, M., Myśliwska, J., & Myśliwski, A. (2009). Ex vivo expansion of $CD4^{+}$ $CD25^{+}$ T regulatory cells for immunosuppressive therapy. *Cytometry Part A*, *75A*(3):175–188. https://doi.org/10.1002/cyto.a.20659

Trzonkowski, P., Bieniaszewska, M., Juścińska, J., Dobyszuk, A., Krzystyniak, A., Marek, N., Myśliwska, J., & Hellmann, A. (2009). First-in-man clinical results of the treatment of patients with graft versus host disease with human ex vivo expanded CD4+CD25+CD127- T regulatory cells. *Clinical Immunology*, 133(1):22–26. https://doi.org/10.1016/j.clim.2009.06.001

van der Windt G.J.W. & Pearce E.L. (2012). Metabolic switching and fuel choice during T-cell differentiation and memory development. *Immunol Rev*, 249(1): 27–42. https://doi.org/10.1111/j.1600-065X.2012.01150.x

Wang, L., Wang, F.-S., & Gershwin, M. E. (2015). Human autoimmune diseases: a comprehensive update. *Journal of Internal Medicine*, 278(4):369–395. https://doi.org/10.1111/joim.12395

Wang, J., Zhao, C., Kong, P., Bian, G., Sun, Z., Sun, Y., Guo, L., & Li, B. (2016). Methylene blue alleviates experimental autoimmune encephalomyelitis by modulating AMPK/SIRT1 signaling pathway and Th17/Treg immune response. *Journal of Neuroimmunology*, 299:45-52. https://doi.org/10.1016/j.jneuroim.2016.08.014

Wu, Z., Jia, X., De La Cruz, L., Su, X.-C., Marzolf, B., Troisch, P., Zak, D., Hamilton, A., Whittle, B., Yu, D., Sheahan, D., Bertram, E., Aderem, A., Otting, G., Goodnow, C. C., & Hoyne, G. F. (n.d.). Article Memory T Cell RNA Rearrangement Programmed by Heterogeneous Nuclear Ribonucleoprotein hnRNPLL. *Immunity*, 29:863–875. https://doi.org/10.1016/j.immuni.2008.11.004

Wu D, Luo Y, Guo W, Niu Q, Xue T, Yang F, Sun X, Chen S, Liu Y, Liu J, Sun Z, Zhao C, Huang H, Liao F, Han Z, Zhou D, Yang Y, Xu G, Cheng T, Feng X (2016) LKB1 maintain Treg Cell Lineage Identity. *Nature Communications*, 8:15876. http://doi.org/10.1038/ncomms15876

Yang K, Blanco DB, Neale G, Vogel P, Avila J, Clish CB, Wu C, Shrestha S, Rankin S, Long L, KC A, Chi H (2017) Homeostasis control of metabolic and functional fitness of Treg cells by LKB1 signaling. *Nature.* 548:602-608. http://doi.org/10.1038/nature23665

Young, K. E., Flaherty, S., Woodman, K. M., Sharma-Walia, N., & Reynolds, J. M. (2017). Fatty acid synthase regulates the pathogenicity of Th17 cells. *Journal of Leukocyte Biology*, 102(5):1229–1235. https://doi.org/10.1189/jlb.3ab0417-159rr

Yurchenko E, Shio MT, Huang TC, Da Silva Martins M, Szyf M, Levings MK, Olivier M, Piccirillo CA (2012) Inflammation-Driven Reprogramming of CD4+Foxp3+ Regulatory T Cells into Pathogenic Th1/Th17 T Effectors Is Abrogated by mTOR Inhibition in vivo. *PLoS ONE,* 7(4): e35572. https://doi.org/10.1371/journal.pone.0035572

Zamoyska, R. (2007). Why Is There so Much CD45 on T Cells? *Immunity*, 27(3): 421–423. https://doi.org/10.1016/j.immuni.2007.08.009

Zanin-Zhorov A, Ding Y, Kumari S, Attur M, Hippen KL, Brown M, Blazar BR, Abramson SB, Lafaille JL, Dustin ML (2010) Protein Kinase C-θ Mediates Negative Feedback on Regulatory T cell Function. *Science*. 328:372-376. http://doi.org/10.1126/science.1186068

Zeiser R, Socié G, and Blazar BR (2016) Pathogenesis of Acute Graft-versus-Host Disease: From Intestinal Microbiota Alterations to Donor T Cell Activation. *British Journal of Haematology.* 175:191-207. http://doi.org/10.1111/bjh.14295.

Zhou, L., Chong, M. M. W., & Littman, D. R. (2009). Plasticity of CD4+ T Cell Lineage Differentiation. *Immunity*, 30(5):646–655. https://doi.org/10.1016/j.immuni.2009.05.001

Zhu, H., Moriasi, C. M., Zhang, M., Zhao, Y., & Zou, M.-H. (2013). *Phosphorylation of Serine 399 in LKB1 Protein Short Form by Protein Kinase C Is Required for Its Nucleocytoplasmic Transport and Consequent AMP-activated Protein Kinase (AMPK) Activation. J. Biol. Chem,* 288:16495-16505. https://doi.org/10.1074/jbc.M112.443580

Zhu, J. (2018). T helper cell differentiation, heterogeneity, and plasticity. *Cold Spring Harbor Perspectives in Biology*, 10:a030338. https://doi.org/10.1101/cshperspect.a030338