The role of LKB1 spliceoforms in iTreg-Th17 plasticity and their interactions with PKCθ and SIRT1 downstream of IL-6 signaling

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The role of LKB1 spliceoforms in iTreg-Th17 plasticity and their interactions with PKCθ, and Sirt1 downstream of IL-6 signaling

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ABSTRACT

The role of LKB1 spliceoforms in iTreg-Th17 plasticity and its interactions with PKCθ, Sirt1 downstream of IL-6 signaling

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Following activation, CD4 T cells undergo metabolic and transcriptional changes as they respond to external cues and differentiate into T helper (Th) cells. T cells exhibit plasticity between Th phenotypes in highly inflammatory environments, such as colitis, in which high levels of IL-6 promote plasticity between regulatory T (Treg) cells and Th17 cells. Protein Kinase C theta (PKCθ) is a T cell-specific serine/threonine kinase that promotes Th17 differentiation while negatively regulating Treg differentiation. Liver kinase B1 (LKB1), also a serine/threonine kinase and encoded by Stk11, is necessary for Treg survival and function. Stk11 can be alternatively spliced to produce a short variant (Stk11s) by transcribing a cryptic exon. However, the contribution of Stk11 splice variants to Th cell differentiation has not been previously explored. Here we show that in Th17 cells, the heterogeneous ribonucleoprotein, hnRNPLL, mediates Stk11 splicing into its short splice variant, and that Stk11s expression is diminished when Hnrnpll is depleted using siRNA knock-down approaches. We further show that PKCθ regulates hnRNPLL
and, thus, *Stk11* expression in Th17 cells. We provide additional evidence that iTreg exposure to IL-6 culminates in *Stk11* splicing downstream of PKCθ. Altogether our data reveal a yet undescribed outside-in signaling pathway initiated by IL-6, that acts through PKCθ and hnRNPLL, to regulate *Stk11* splice variants and induce Th17 cell differentiation. Furthermore, we show for the first time, that this pathway can also be induced in iTregs exposed to IL-6, providing mechanistic insight into iTreg phenotypic stability and iTreg to Th17 cell plasticity. Sirtuin 1 (Sirt1) plays a crucial role in metabolism and inflammatory responses. Sirt1 is a deacetylase that can regulate different transcription factors important for modulating immune responses. Sirt1 has been shown to increase iTreg conversion to Th17 cells by deacetylating the *Foxp3* transcription factor in iTregs. LKB1, encoded by *Stk11*, has been identified as a mediator of Treg induction and function through its effects on TSDR methylation and metabolism. Sirt1 has been shown to activate LKB1, but the molecular mechanisms of these signaling pathways are not known. Our data suggest *Stk11* splicing may lie at the heart of iTreg responses to IL-6 exposure and may represent a unique target for developing stable, cell-based iTreg therapies. Examining the contribution of Sirt1 to this process may provide additional insight as to how LKB1 isoforms regulate iTreg-Th17 cell fate decisions.
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CHAPTER 1

INTRODUCTION

1.1 The immune system

The immune system is a complex network of organs and proteins that defends the body against infections while protecting itself. The immune system consists of different types of bone marrow-derived (hematopoietic) cells that have evolved to recognize and react to foreign substances, known as antigens (Janeway, 1992; Medzhitov, 2007). The immune system can detect and respond to a wide array of pathogens stemming from parasites and worms, cancer cells and objects such as a splinter, distinguishing them from the organisms own healthy tissues. The immune system can be divided into two parts, the innate and adaptive immune systems (Figure 1.1) (Vivier & Malissen, 2005). The innate immune system, also known as, nonspecific immunity, is the defense system you are born with protecting against all antigens. These barriers are a first line of defense that get established within minutes after the onset of the infection. Innate defense mechanisms consist of physical barriers or epithelia (e.g., skin, oral mucosa, respiratory epithelium, intestine), as well as circulating antimicrobial proteins, and innate immune cells. Innate immune cells include granulocytes, macrophages, dendritic cells, innate lymphoid cells, and natural killer cells, which recognize a wide variety of pathogen- or damage-associated molecules (Akira et al., 2006; Bird et al., 2018).

The second line of defense is the adaptive immune system, which is unique to vertebrates, and encompasses B and T cells. The adaptive immune system express
surface receptors that specifically recognize individual immune determinants, or epitopes, on antigens (Burnet, 1959; Pancer & Cooper, 2007). Unlike the innate immune system which is pre-programmed to react to common broad categories of pathogen, the adaptive immune system is highly specific to each pathogen the body has encountered. Adaptive immunity gets activated by antigen-presenting cells, such as dendritic cells, when pathogens resist to innate immune responses, and participates in pathogen elimination by using processes that specifically target antigen-expressing pathogens (Qu & Chaiko, 2010; Iwasaki & Medzhitov, 2015). B cells develop in the bone marrow and contribute to immune responses through the production of antibodies (humoral immunity), while T cells mature in the thymus and secrete effector molecules and cytokines which contribute either directly or indirectly to pathogen clearance (cellular immunity) (Waksman et al., 1962; Ryser & Vassalli, 1974; Spits, 2002; Cooper, 2015).

Unlike the innate immune system, adaptive immune responses are slower, as adaptive immune cells need to differentiate and acquire their function after responding to presented antigen. However, the duration of these responses lasts longer and results, after the resolution of an infection, in the formation of long-lived memory cells. These memory cells respond faster, with higher affinity, and more efficiently upon subsequent exposures to the same antigen (Figure 1.2) (Hammarlund et al., 2003).

1.2 Generation of T cells

T cell development occurs in the thymus. The thymic microenvironment differentiation as well as positive and negative selection. In the thymus, T cells develop their specific T cell markers, including TCR, CD3, CD4 or CD8, and CD2. T cells also undergo thymic education through positive and negative selection. The thymus is a multi-
lobed structure composed of two parts, cortical and medullary regions. These areas are surrounded by a capsule. T cell precursors enter the subcapsular cortical areas, where they encounter networks of cortical epithelial cells (the thymic stroma) and undergo proliferation (Kumar et al., 2018). As these T cells differentiate, they move from the cortex towards the medulla of the thymus, different microenvironments within the thymus direct T cell development (Kumar et al., 2018). Most cells that enter the thymus die by apoptosis without successfully completing the steps required for becoming a mature naive T cell (Kumar et al., 2018).

In the initial stages, progenitor cells have not undergone TCR rearrangement for genes CD2+CD3− but begin to express CD2, thus they are double negative for CD4 or CD8, T helper and cytotoxic T cell markers lineage. These cells next express the adhesion molecule CD44, then a chain of the IL-2 receptor (CD25) (Farber et al., 2014). CD44 low CD25+ double negative T cells rearrange TCR β chain. Beta chain rearrangement begins with D-J joining, followed by V-DJ joining (Farber et al., 2014). The chances of successful β chain rearrangement are increased by the presence of two DJCb gene clusters (Farber et al., 2014). If rearrangement in the first cluster fails, rearrangement in the second can occur.

Double positive αβ TCR low cells must undergo positive and negative selection before they leave the thymus. Cells that have successfully rearranged the TCR will die in the thymus cortex if they do not bind self MHC within 3-4 days (Farber et al., 2014). These cells can undergo either positive or negative selection. Positive selection occurs when double positive T cells bind cortical epithelial cells expressing Class I or Class II MHC plus self-peptides with a high enough affinity to get the survival signal (Xiong & Bosselut,
If TCRs are incapable of binding, the T cell will undergo a type of cell death called apoptosis. Further, this positive selection process also determines if a T cell will become a CD8+ T cell or a CD4+ T cell (Xiong & Bosselut, 2012). Specifically, if a TCR complex binds strongly to MHC class II, the complex will send intracellular signals to induce the expression of a protein called ThPOK (Xiong & Bosselut, 2012; Wu et al., 2022). This protein reduces the expression of another key protein, called Runx3, responsible for driving CD8 expression (Wu et al., 2022). Because low Runx3 causes low CD8 expression, these ThPOK+, Runx3- cells become CD4+ T cells. If, however, a developing T cell does not bind strongly to MHC class II, ThPOK levels will be low and thus Runx3 levels will be high, pushing the T cell to differentiate into a CD8+ cell (Wu et al., 2022). Thus, the process of positive selection leads to the survival and expression of mature CD8+ and CD4+ T cells capable of recognizing MHC complexes. While the ability of T cells to recognize antigen-MHC complex is vital for their ability to fight pathogens and other foreign cells, it is equally important that these T cells do not recognize and attack our own cells. This is where negative selection comes into play. Negative selection occurs when double positive T cells bind to bone-marrow derived APC (macrophages and dendritic cells) expressing Class I or Class II MHC plus self-peptides with a high enough affinity to receive an apoptosis signal, thereby eradicating immature T cell that have a high likelihood of being self-reactive (Xiong & Bosselut, 2012) (Figure 1.3).

The goal of T cell development in the thymus is, thus, to produce a functional pool of mature MHC-restricted and self-tolerant mature T cells with distinct differentiation potentials. After thymic education, mature T cells exit the thymus and migrate to secondary lymphoid organs, such as the spleen and lymph nodes (McCaughtry et al., 2007).
1.3 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, and differentiation are discussed below:

1.3.1 Activation of Naïve T cells

To be activated, a naive T cell must recognize a foreign peptide bound to a self MHC molecule. But this is not, on its own, sufficient for activation. That requires the simultaneous delivery of a co-stimulatory signal by a specialized antigen-presenting cell. Only dendritic cells, macrophages, and B cells are able to express both classes of MHC molecule as well as the co-stimulatory cell-surface molecules that drive the clonal expansion of naive T cells and their differentiation into armed effector T cells (Tai et al., 2018).

T cells require three signals to become fully activated. Signal One: T cells are generated in the thymus and are programmed to specifically recognize one foreign particle (antigen). Once they leave the thymus, they circulate throughout the body until they recognize their antigen on the surface of antigen presenting cells (APCs) (Tai et al., 2018). The T cell receptor (TCR) on CD4+ helper T cells and CD8+ cytotoxic T cells binds to the antigen as it is held in a structure called the MHC complex, on the surface of the APC. This triggers initial activation of the T cells (Figure 1.4). Signal two: In addition to TCR binding to antigen-loaded MHC, both helper T cells and cytotoxic T cells require several secondary signals to become activated and respond to the threat. In the case of helper T cells, the first of these is provided by CD28. This molecule on the T cell binds to one of two molecules on the APC – B7.1 (CD80) or B7.2 (CD86) – and initiates T-cell proliferation. This
process leads to the production of many millions of T cells that recognize the same antigen (Figure 1.4). Signal three: Once the T cell has received a specific antigen signal and a co-stimulatory signal two, it receives more instructions in the form of cytokines. These determine which type of responder the cell will become (Figure 1.4). The resulting cell population moves out to the site of the infection or inflammation to deal with the pathogen (Tai et al., 2018).

The interactions between TCR:MHC and CD28:CD80/86 will promote the formation of an immunological synapse between the T cell and APC. An immunological synapse consists of molecules involved in T cell activation. 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).

1.3.2 Cytokine Signaling

Cytokines represent a diverse group of molecules that transmit intercellular signals. These signals may either be autocrine (where the same cell both produces the cytokine and responds to it) or paracrine (where the cytokine is made by one cell and acts on another). Both these situations can occur simultaneously (Leonard & Lin et al., 2000). Cytokines use
multiple signaling pathways. The Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway plays critical roles in orchestrating immune responses, especially through cytokine receptors, as they can modulate the polarization of T helper cells (Seif et al., 2017).

JAK-STAT signaling occurs when JAK, a tyrosine kinase causes the phosphorylation and autophosphorylation of the intracellular domain of the cytokine receptor. The associated JAK combination phosphorylates the receptor cytoplasmic domain to allow recruitment of a STAT, which in turn is phosphorylated, dimerizes, and moves to the nucleus to bind specific sequences in the genome and activate gene expression. Cytoplasmic domains of cytokine receptors associate with JAKs via JAK binding sites located close to the membrane (Murray, 2007). JAK-mediated phosphorylation of the receptor creates binding sites for the Src homology 2 (SH2) domains of the STATs (Behrmann et al., 2004). STAT recruitment is followed by tyrosine, and in some cases, serine phosphorylation on key residues (by the JAKs and other closely associated kinases) that leads to transit into the nucleus (Behrmann et al., 2004).

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 1.5.

1.3.3. CD4+ helper T cells
Activated CD4 T cells differentiate into helper T cells or regulatory T cells to elicit specific immune responses to different types of pathogens (Zhu, 2018). There are several different helper T cell subsets which perform different functions. The main subsets are depicted in Figure 1.6. These include T helper type 17 (Th17) cells, follicular helper T cells (Tfh), induced T regulatory cells (iTreg), T helper type 2 cells (Th2), as well as the potentially distinct T helper type 9 (Th9) cells. 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, 2018). In this thesis two main subsets of CD4 T cells were focused on and discussed below.

1.3.3.1 Th17 Cell Differentiation and Function

IL6, IL21, IL23, and TGFβ are the major signaling cytokines involved in Th17 cells differentiation, and retinoic acid receptor-related orphan receptor gamma-T (RORγt) is the master regulator (Luckheeram et al., 2012).

TGFβ is the critical signaling cytokine in Th17 cell differentiation (Veldhoen et al., 2006; Mangan et al., 2006; Bettelli et al., 2006; Manel et al., 2008; Volpe et al., 2008). However, TGFβ signaling pathways also play significant roles in the development of iTreg cells. Both Th17 and iTreg cells have opposing functions but are antagonistically related. At high concentration, TGFβ can divert T cell lineage differentiation towards iTreg cell development, through the induction of Foxp3 (Chen et al., 2003; Zhou et al., 2008). However, at low concentration and in the presence of IL6, TGFβ induces Th17 cell differentiation, and leads to the production of IL17 and IL21 cytokines (Veldhoen et al.,
TGFβ together with IL6 leads to the activation of RORγt, a master transcription factor of Th17 cells.

STAT3, activated downstream of IL6, IL21, IL23 signaling, plays an important role in the differentiation process, as it induces RORγt expression. STAT3 deficiency was found to cause enhanced expression of T box expressed in T cells (Tbet) and Forkhead transcription factor 3 (Foxp3), which are involved in the development of opposing cell lineages (Yang et al., 2007). STAT3 binds to Il17a and Il17f promoters (Chen et al., 2006).

RORα, another member of the ROR family, also participates in the lineage commitment pathway. Together, RORα and RORγt synergistically enhance Th17 cell differentiation, and their absence completely abrogated the development of Th17 cells (Yang et al., 2007). Runx1 also influences Th17 differentiation through the induction of RORγt.

Literature suggests Th17 cells are not only critical for maintaining mucosal immunity, but their dysregulation has been implicated in the pathogenesis of autoimmune inflammation. Th17 cells have since been demonstrated to play a role in the progression of other autoimmune diseases, such as rheumatoid arthritis, psoriasis, multiple sclerosis, and inflammatory bowel disease (Aswathi & Kuchroo, 2009). The Th17 cytokines IL17A and IL17F trigger the production of pro-inflammatory cytokines in target tissues, which not only mediate inflammation through the recruitment of innate immune cells such as neutrophils, but also promote further Th17 cell activation through a positive feedback mechanism (Aswathi & Kuchroo, 2009). The precise environmental cues that lead to Th17 cell differentiation determine whether they develop towards a tissue-protective or
pathogenic phenotype, as research suggests that differentiation triggered by high levels of IL6 and IL1β results in a more pro-inflammatory role (Yang et al., 2008). Currently, studies are focused on understanding the role of Th17 cells and their cytokine products as therapeutic targets for autoimmunity.

1.3.3.2 Regulatory Cell Differentiation and Function

Regulatory T cells (Tregs) are crucial for limiting the severity of the immune response and preventing the attack on self-tissue. Naturally occurring Tregs (nTregs) arise from the thymus or can be induced in the periphery (iTregs). 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. TGFβ is the critical cytokine responsible for the initiation of the iTreg cell lineage commitment (Chen et al., 2003; Li et al., 2007). Foxp3 is specifically expressed in CD4+CD25+Treg cells and is the major lineage-specific transcription factor involved in iTreg cell differentiation (Yagi et al., 2004). Foxp3 mutations lead to the immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome which causes widespread autoimmunity due to Treg cell deficiency. Foxp3 expression is regulated at its promoter through three conserved, noncoding 5 sequence (CNS) regions (Fontenot et al., 2003).

Smad2 and Smad3, which are also activated through TGFβ signaling pathways, are involved in the iTreg cell differentiation process by inducing FOXP3 (Takimoto et al., 2010). Smad3 can differentially enhance iTreg cell development by upregulating Foxp3 expression and inhibit Th17 differentiation by blocking RORγt (Martinez et al., 2009). STAT5, induced downstream of IL2 signaling, is required for the differentiation of iTreg cells (Laurence et al., 2007). STAT5 was found to enhance Foxp3 expression and,
subsequently downstream to Foxp3 signaling, promote iTreg cell development (Laurence et al., 2007).

Tregs cells’ primary effector cytokines include IL10, TGFβ, and IL35. IL10 is a potent inhibitory cytokine, with the ability to suppress proinflammatory response and, thus, limit the tissue damage caused by the inflammatory process (Couper et al., 2007). IL10 and TGFβ potently suppress IgE production, thereby highlighting their important roles in attenuating allergic inflammation (Jutel & Akdis, 2008). 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).

1.4 T cell motility and migration to tissues

T cell migration is essential for T cell responses. T cell migration allows for the detection of cognate antigen at the surface of antigen-presenting cells and for interactions with other cells involved in immune response. T cell motility patterns are governed by mechanisms that are optimized both for the activation status of the cell and environment-specific cues. T cell motility arises from the combination of three distinct processes: autonomous cell locomotion, physical guidance, and response to chemical signals (Krummel et al., 2016).

Autonomous cell locomotion depends on the cell-intrinsic motility machinery, including the actin cytoskeleton and motor proteins, while physical guidance is controlled by interactions with the extracellular matrix and surrounding cells. The response to chemical signals relies on the expression of 13 chemokine receptors on the T cell surface (Figure 1.6). Depending on the stage of T cell differentiation, its motility can be divided
into three parts. Naïve T cells migrate to lymphoid organs and use a diffusive (e.g., Brownian-type) or sub-diffusive random walk (Krummel et al., 2016). Recently activated T cells use chemotaxis signals which are sent by cognate APCs, most likely to attract these T cells to different environments (Krummel et al., 2016). Effector T cells migrate to peripheral sites using either diffusive or super-diffusive random walks.

Chemokines and their associated receptors are key modulators of T cell migration. Chemokines are a large group of small, structurally related cytokines that function as chemo-attractants and play fundamental roles in T cell biology by directing cell migration through interactions with chemokine receptors (Aaron et al., 2022). For example, mature naive T cells traveling in the bloodstream express CCR7, the receptor that recognizes and guides these cells towards gradients of the chemokines CCL19 and CCL21. These chemokines are secreted by stromal cells of the secondary lymphoid organs, such as the spleen and lymph nodes, where antigen presentation occurs (Gunn et al., 1998; Förster et al., 1999; Luther et al., 2000). T cells constantly interact with endothelial cells through repeated bind-release events between adhesion molecules, such as L-selectin (CD62L), and their ligands, a process termed “rolling” (Kishimoto et al., 1990). T cells that respond to CCL19/CCL21 upregulate surface adhesion molecules that will bind more strongly to the endothelium and undergo rearrangement of their actin cytoskeleton to promote the transmigration of these cells from the bloodstream into the secondary lymphoid organs through specialized blood vessels, high endothelial venules (HEV) (Stein et al., 2000). Many cell types at sites of ongoing immune responses secrete chemokines that will also generate gradients. This results in the recruitment of the appropriate types of effector T cells, which will be specifically responsive to these signals through the expression of the
corresponding cognate chemokine receptors (Kagnoff & Eckmann, 1997; Griffith et al., 2014). Once in the inflamed tissue, effector T cells keep following chemokine gradients, and arrest upon antigen re-encounter.

1.5. T cell metabolism

T cell activation leads to dramatic shifts in cell metabolism to protect against pathogens and to orchestrate the action of other immune cells. Further, functionally distinct T cell subsets require distinct energetic and biosynthetic pathways to support their specific functional needs. Pathways that control immune cell function and metabolism are intimately linked, and changes in cell metabolism at both the cell and system levels have been shown to enhance or suppress specific T cell functions. Thus, cell metabolism is now appreciated as a key regulator of T cell function, specification, and fate. In the next section, preferences in energy metabolism for naïve, effector, and regulatory T cells will be discussed (Figure 1.7).

1.5.1 Metabolism of the naïve T cell

Naïve T cells are metabolically quiescent until they encounter antigen. Naïve T cells rely on the full oxidation of glucose through OXPHOS, in the absence of TCR stimulation (Rivera et al., 2021). These T cells can only survive when homeostatic cytokines, like interleukin 7 (IL-7) provide signaling cues (Rathmell et al., 2000). This pathway, in naïve T cells promotes the translocation of the glucose transporter 1 (Glut1) to take up glucose (Rivera et al., 2021). Glucose is then broken down into pyruvate, a substrate that enters the mitochondria to activate the synthesis of triacyl glycerol, which serves as a source of lipids that fuels into the fatty acid oxidation (FAO) pathway (Wofford et al., 2008; Edinger et al., 2002). In contrast to this maintenance phase, many nutrients
(glucose, glutamine, Larginine, and other amino acids) are needed to differentiate naïve T cells into the effector phenotype upon antigen encounter (Bental et al., 1993).

### 1.5.2 Glycolysis in Effector T Cell Function

Upon activation, naïve T cells undergo a process known as the “Warburg Effect”, depicted in Figure 1.8, which changes the cell’s primary means of energy production from catabolism to anabolism (van der Windt and Pearce, 2012). Warburg metabolism, initially discovered as an important pathway for the survival of malignant cells, is characterized by an extraordinary ability to breakdown glucose by anaerobic glycolysis and amino acids such as glutamine (Warburg & Wind, 1927). 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 converts glucose to lactate via the enzyme lactate dehydrogenase (LDHA) under normal oxygen conditions. A byproduct of this lactate production is NAD+, which is an electron carrier used in glycolysis and OXPHOS. Other byproducts of this process such as 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 also produces 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 co-stimulation can increase T cell anabolic metabolism, while the CD28 family members PD-1 and CTLA4 suppress T cell metabolic reprogramming (Beckermann et al., 2020). CD28 signal during initial T cell activation helps prime mitochondria with elevated spare respiratory capacity, that is necessary for the rapid recall responses of memory T cells (Klein Geltink et al., 2017). 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. mTORC1 will also activate Hypoxia Inducible Factor 1α (HIF1α) which upregulates GLUT1 transcription. 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.
1.5.3 Metabolic processes of regulatory T cells

Tregs differ in their metabolic requirements as compared to helper T cells and naïve T cells. Studies have revealed that expression of Foxp3 reprograms T cell metabolism by suppressing glycolysis and enhancing OXPHOS (Angelin et al., 2017, Gerriets et al., 2016). The effector molecules CTLA4 and PD1 on Treg cells also suppress glycolysis in T cells (Patsoukis et al., 2015, Parry et al., 2005). Several studies have indicated that elevated glycolysis may be detrimental to Treg cell induction, as inhibition of glycolysis promotes the induction of Foxp3 expression in response to TGFβ and IL-2 stimulation (Eleftheriadis et al., 2013, Shi et al., 2011). In addition, deletion of HIF-1α, a transcription factor that can promote glycolysis, also leads to increased Foxp3 induction (Shi et al., 2011).

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). 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. Although Treg cells mainly use oxidative metabolism
for growing, Treg proliferation is dependent on an oscillatory switch of glycolysis (Procaccini et al., 2010).

1.5.4 Metabolism and plasticity between iTregs and Th17

Th17 and iTregs are plastic in their nature and can change phenotypes. Th17 and Treg cells regulate the differentiation of one another to maintain equilibrium. Both T cell lineages require TGFβ for their differentiation. TGFβ induces the expression of Foxp3 in naïve T cells, promoting the development of Tregs, but in the presence of IL6, it inhibits the generation of Tregs while it induces the expression of RORγt, thus promoting a Th17 phenotype. In addition, TGFβ-induced Foxp3 expression negatively regulates Th17 cell differentiation by repressing RORγt expression. Retinoic acid has also been involved in the Treg-Th17 balance; it can induce Foxp3 and inhibit RORγt even under Th17-inducing conditions, promoting the development of Tregs. In addition, dual Th17/Treg precursors have been found, which express both RORγt and Foxp3 simultaneously, that can commit to one or the other subset. This inter-dependent regulation of Th17/Treg differentiation is critical to combine immune homeostasis and pathogen clearance. In several autoimmune diseases such as psoriasis and arthritis, in vivo iTregs can take on Th17 functions such as IL17 production (Figure 1.9). It has been shown, in vitro and in vivo, that Th17 cells can be reprogrammed into FOXP3+ T cells, and these correlate with increased disease survival. Plasticity between iTreg and Th17 cells has shown iTregs to adopt a proinflammatory state which has been demonstrated in autoimmune conditions such as colitis. Identifying key molecules that regulate iTreg-Th17 plasticity will aid in developing therapeutics to prevent iTregs from adopting a pro-inflammatory state.
TGFβ is a key regulator of the observed plasticity that exists between Tregs and Th17 cells. In response to TGFβ 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 TGFβ is accompanied by signaling initiated by the proinflammatory cytokine IL6, Foxp3 is downregulated, allowing the induction of the Th17 program (Ren and Li, 2017; Zhou et al., 2009).

FOXP3+ Treg cells are functionally and phenotypically stable after many cell divisions in inflammatory sites (Rubtsov et al., 2010). This suggests that stability of Tregs is maintained by types of constant cell-extrinsic stimuli (such as antigen, co-stimulation, and cytokines) and/or by a cell-intrinsic mechanism that induces Treg cells to express Treg function-associated molecules. As seen in the process of Th cell differentiation from naive T cells after antigen exposure, Treg cells show adaptive properties by sensing environmental cues (Koch et al., 2009). For example, like Th1 cells, Foxp3+ Treg cells that are recruited to a site of Th1-type inflammation express Tbet and CXC-chemokine receptor 3 (CXCR3), and they apparently produce interferon γ (IFNγ) (Koch et al., 2009). Similarly, Treg cells expressing STAT3, RORγt, and CC-chemokine receptor 6 (CCR6) as well as producing IL17, accumulate at sites of Th17 cell-mediated inflammation (Chaudhry et al., 2009). However, it is unclear whether proinflammatory cytokine signaling causes Tregs to become ex-Treg cells or to improve their suppressive capacity. It is thought that IL2 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 ex-Tregs (Raffin et al., 2019).
The most striking difference between iTreg and Th17 cells are their metabolic phenotypes. The Th17 cells exhibit the most glycolytic phenotype of all the T helper subsets, while the iTregs employ processes vastly different from all the other T helper metabolism phenotypes. Th17 cells use glycolysis, the Pentose Phosphate Pathway, OXPHOS, the hexosamine pathway, and FAS, whereas iTregs utilize catabolic processes, mainly FAO, as their energy source. Identifying regulators of metabolic status may lay the foundation for developing therapeutics to modulate the immune response.

1.6. The role of LKB1 in T cells

1.6.1 An overview of LKB1 pathway

Liver kinase B1 (LKB1) is a serine/threonine kinase that mediates key processes including cellular energetics and growth. It was first identified as a tumor suppressor that, when mutated, caused spontaneous tumors such as those found in Peutz-Jeghers syndrome (Lochner et al., 2015). LKB1 functions in energy-sensing by activating 5’ adenosine monophosphate protein kinase (AMPK) to further initiate fatty acid oxidation by inhibiting mTORC1 and activation of the fatty acid oxidation enzyme carnitine palmitoyltransferase 1a (CPT1a), a fatty acid oxidation enzyme (Ma et al., 2017; He et al., 2017). The human LKB1 protein comprises 433 residues and consists of the N-terminal noncatalytic domain, the two nuclear localization signals, the kinase domain, and the C-terminal regulatory domain (Kuwako & Okano, 2018). The activation of LKB1 is allosterically controlled by two cofactors, the LKB1-binding pseudokinase STE20-related adaptor (STRAD) and the scaffolding protein MO25. STRAD directly binds to LKB1, and this interaction is facilitated and stabilized by MO25, which binds to the C-terminal of STRAD (Baas et al.,
2003; Boudeau et al., 2003). Once STRAD binds to LKB1, LKB1 is activated and translocated from the nucleus to the cytoplasm to exert its functions.

LKB1 is encoded by the gene Stk11, located on chromosome 19p. Alternative splicing of pre-mRNA is a mechanism which results in multiple transcript variants of a single gene. In humans, two STK11 isoforms have been reported, an alternatively spliced isoform which has variation at its C-terminal and mostly expressed in testis (LKB1s). Another isoform exhibiting oncogenic properties lacks several residues at its N-terminal (ΔN-LKB1). Stk11 has also been reported to be alternatively spliced into a long form. 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) (Figure 1.10).

Once Stk11 is translated into protein, it has a nuclear localization sequence on the N-terminus, followed by the kinase domain. The C-terminus has modification sites for phosphorylation and farnesylation. LKB1(S) is a novel form in which the last 63 residues are replaced by a unique 39-residue sequence lacking known phosphorylation (Ser431) and farnesylation (Cys433) sites. 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). Although these isoforms exhibit differences in structure, they 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 C ζ 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. Thus, alternative splicing of \textit{Stk11} gene generating new variant with heterogeneous properties suggests for complex regulation of these variants in controlling the AMPK pathway and other functions.

\textbf{1.6.2 LKB1 in effector T cells}

Lymphocyte metabolism is a highly regulated process that plays a key role in the determination of T cell fate and function, and LKB1 is an established regulator of cell metabolism (Maclver et al., 2011). However, while LKB1 has been shown to be important for thymocyte development, its role in peripheral T cell metabolism, and effector function is not well defined. Activated effector T cells rely heavily on glucose metabolism to survive, proliferate, and support specific effector functions (Rathmell et al., 2000; Jacobs et al., 2008). Maclver and his colleagues investigated the influence of LKB1 loss in T cells. They found that LKB1\textsuperscript{-/-} T cells have increased glycolytic rates and elevated expression of glycolytic enzymes (Maclver et al., 2011). Additionally, in the absence of LKB1, the uptake of glucose is increased due to increased expression of the glucose transporter, GLUT1. LKB1\textsuperscript{-/-} T cells also show increased activation as evidenced by CD44 expression and greater cytokine production. When LKB1\textsuperscript{-/-} CD4 T cells were polarized in vitro towards a Th1, Th2, or Th17 phenotype, all subsets showed increased cytokine production as compared to WT cells. This phenomenon seemed to be unique to LKB1, as AMPK\textsubscript{\alpha}{\textsuperscript{-/-}} 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 IL17A and IFN\textgamma; however, this effect was mirrored in AMPK deficient CD8 T cells. Interestingly, the increased levels of IFN\textgamma in both the LKB1\textsuperscript{-/-} and AMPK\textsubscript{\alpha}{\textsuperscript{-/-}} 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).

One of the targets that links LKB1 to cellular metabolism is the serine/threonine kinase AMP-activated protein kinase (AMPK), thus LKB1 is essential for AMPK activation under conditions of bioenergetic stress (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 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, due to inadequacy of the energy demands put forth by rapid proliferation. These cells expressed higher amounts of Bax, a proapoptotic BCL2 family protein, than wild type cells, upon metabolic stress. However, this survival defect can be circumvented by transgenic expression of BcL-xL (MacIver et al., 2011).

1.6.3 LKB1 in regulatory T cells

Tregs play a pivotal role in establishing and maintaining self-tolerance and homeostasis. Yang and his colleagues investigated how LKB1 aids Treg cells orchestrate their homeostasis and interplay with environmental signals. Mice with a Treg-specific deletion of LKB1 developed a fatal inflammatory disease characterized by excessive Th2-type-dominant responses. LKB1 deficiency disrupted Treg cell survival and mitochondrial fitness and metabolism. LKB1 function in Treg cells was independent of conventional AMPK signaling or the mTORC1–HIF-1α axis. Thus, Treg cells use LKB1 signaling to coordinate their metabolic and immunological homeostasis and to prevent apoptotic and
functional exhaustion, orchestrating the balance between immunity and tolerance (Yang et al., 2017). As expected, LKB1 deficiency reduced 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 possibly suggests 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). Other ways LKB1 has been shown to promote homeostasis and aid in stability of Tregs is through the mevalonate pathway. LKB1-deficient Treg cells demonstrate reduced activity of the mevalonate pathway which lead to reduced expression of Foxp3 and subsequently less suppressive capacity. LKB1-deficiency and subsequent aberration of the mevalonate pathway led to the Tregs expressing IFN\(\gamma\) and IL17A, suggesting phenotypic instability (Timilshina et al., 2019).

Several studies have shown that ablation of LKB1 in Tregs induces widespread autoimmunity. These effects 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. Their results showed 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 TGFβ signaling as LKB1 floxed Tregs have reduced TGFβ signaling due to the lower expression of TGFβ receptors (Wu et al., 2017).

1.7 PKCθ in T cell signaling

1.7.1 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^{2+}- 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 synapase created between the T cell and APC. Recruitment of PKCθ to the immunological synapase allows for the interaction between PKCθ and LCK and LCK acts as a bridge PKCθ and CD28 and it plays an integral role in activating a range of signaling cascades that results in transcriptional network in T cells (Brezar et al., 2015).

Protein kinase C is a kinase family that has different isoforms activated either by proteolysis or translocation to plasma membrane, where it binds to specific cofactors. Diacylglycerol (DAG) and inositol 1, 4, 5-trisphosphate (IP3) are the cofactors that activate PKC enzymes (Isakov & Altman, 2012; Takai et al., 1997). These enzymes transduce signals from cell surface receptors that are associated with phospholipase C and phospholipid hydrolysis. This family includes 3 subfamilies and 10 isoforms that are structurally and functionally related (Pfeifhofer-Obermair et al., 2012). The first subfamily
contains conventional PKCs (cPKC; α, βI, βII, γ) that are regulated via DAG binding C1 domains and Ca+2 binding (Ho et al., 2001). The second group includes novel PKCs (nPKC; δ, ε, η, θ) that are DAG-dependent but Ca+2-independent for their activity. The third group has atypical PKCs (aPKC; ζ, λ, ι) that are both DAG- and Ca+2-independent (Rosse et al., 2010). Immunological studies show that distinct PKC isoforms have different ways of activating function of T cells and thus, they can serve as drug targets for T cell mediated adaptive immune responses (Yokosuka et al., 2008).

Among the PKC enzymes, protein kinase C-theta (PKCθ) exhibits a selective pattern of tissue distribution with a predominant expression in T lymphocytes, platelets, and skeletal muscle. It is translocated to the center of immunological synapse (IS) in activated T cells where the integration of T cell receptor (TCR) and CD28 costimulatory signal occurs for its full activation (7). Upon its full activation, three transcription factors including NFκB, AP-1, and NFAT initiate signals that are critical for T cell activation, proliferation, and differentiation (Blagih et al., 2012). Recently, it has been suggested that PKCθ can also translocate to the nucleus and binds to chromatin to regulate microRNA and T cell-specific gene expression programs (Figure 1.11). PKCθ mediates the signals required to stimulate Stat3 expression which is critical for Th17 differentiation. Th17 differentiation was greatly inhibited in PKCθ−/− T cells, and this was accompanied by reduced levels of STAT3. Taken together, these results from the study indicates that PKCθ stimulates Th17 differentiation.

PKCθ regulates the activity of NF-kB upon T cell activation which culminates in IL2 production. NFκB 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 NFκB. 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 NFκB transcription factors from the cytoplasm (Isakov and Altman, 2002; Lucas et al., 2004; Brezar et al., 2015). 27 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).

1.7.2 PKCθ has opposing functions in effector and regulatory T cells

Studies have shown that PKCθ is crucial for Th17 cell differentiation as PKCθ-/- T cells exhibit a diminished Th17 phenotype. Th17 and Treg cells regulate the differentiation of one another to maintain equilibrium. Both lineages require TGFβ for their differentiation. TGFβ induces the expression of Foxp3 in naïve T cells, promoting the development of Tregs, but in the presence of IL6, it inhibits the generation of Tregs while it induces the expression of RORγt, thus promoting a Th17 phenotype (Figure 1.12). There are several Th17 differentiation mechanisms that require PKCθ function. Downstream of IL6, STAT3 is activated and induces the upregulation of RORγt and IL17. PKCθ has been shown to regulate Stat3 expression, (Kwon et al., 2013). SRC1 promotes Th17 differentiation by overriding Foxp3
suppression to stimulate RORγt activity in a PKCθ-dependent manner. PKCθ is crucial for Th17 cell differentiation and effector function, through modulation of IL17 production (Sen et al., 2018).

In Tregs, PKCθ has been shown to have an inhibitory role especially to its suppressive activity. PKCθ−/− mice exhibit increased Treg number in vivo, and an increased capacity for in vitro Treg differentiation. Furthermore, knockdown or over expression of the AKT downstream targets FoxO1 and FoxO3a was found to inhibit or promote iTreg differentiation in PKCθ−/− T cells, accordingly, indicating that the AKT-FoxO1/3A pathway is responsible for the inhibition of iTreg differentiation downstream of PKCθ (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 (TNFα) production. This has been shown to prevent the Treg from undergoing TNFα-mediated inactivation. Furthermore, in autoimmune models, if PKCθ is inhibited in the Treg cells, the Treg cells are more efficacious in suppression in the proinflammatory autoimmune environment (Zanin-Zhorov et al., 2010).

1.8 RNA binding proteins

1.8.1 RNA splicing and proteins

The initial RNA that is transcribed from a gene's DNA template must be processed before it becomes a mature messenger RNA (mRNA) that can direct the synthesis of protein. Before the translation to protein, one of the steps in this process that occurs is called RNA splicing. This involves the removal or "splicing out" of certain
sequences referred to as intervening sequences, or introns. The final mRNA consists of the remaining sequences, called exons, which are connected to one another through the splicing process. RNA splicing was initially discovered in the 1970s, overturning years of thought in the field of gene expression.

Maturation of the RNA allows for its export from the nucleus to the cytoplasm to the ribosome for translation. There are three major modifications that occur during splicing: Three major modifications occur during the maturation process: 5’ capping, 3’ polyadenylation, and splicing (Bentley, 2014). The capping process protects the 5’ end of the primary RNA transcript from attack by ribonucleases that have specificity to the 3’5’ phosphodiester bonds. As the poly(A) tail is synthesized, it binds multiple copies of poly(A) tail-binding protein which protects the 3’end from ribonuclease digestion by enzymes. The third modification is intron splicing during which introns regions of RNA that do not code for proteins, are removed from the pre-mRNA and the remaining exons connected to re-form a single continuous molecule. In addition to the basic splicing out of introns, alternative splicing can also occur. Alternative splicing is a cellular process in which exons from the same gene are joined in different combinations, leading to different, but related, mRNA transcripts. Alternative splicing introduces more diversity by changing the established intron boundaries in the transcript. These allow for the different versions, or isoforms, of the same transcript (Black et al., 2019).

Splicing occurs in several steps and is catalyzed by small nuclear ribonucleoproteins (snRNPs, commonly pronounced "snurps") (Clancy, 2008). First, the pre-mRNA is cleaved at the 5’ end of the intron following the attachment of a snRNP called
U1 to its complementary sequence within the intron (Clancy, 2008). The cut end then attaches to the conserved branch point region downstream through pairing of guanine and adenine nucleotides from the 5’ end and the branch point, respectively, to form a looped structure known as a lariat (Clancy, 2008). Next, the snRNPs U2 and U4/U6 appear to contribute to positioning of the 5’ end and the branch point in proximity. With the participation of U5, the 3’ end of the intron is brought into proximity, cut, and joined to the 5’ end. The adjoining exons are covalently bound, and the resulting lariat is released with U2, U5, and U6 bound to it (Clancy, 2008). In addition to consensus sequences at their splice sites, eukaryotic genes with long introns also contain exonic splicing enhancers (ESEs) (Clancy, 2008). These sequences, which help position the splicing apparatus, are found in the exons of genes, and bind proteins that help recruit splicing machinery to the correct site. Most splicing occurs between exons on a single RNA transcript, but occasionally trans-splicing occurs, in which exons on different pre-mRNAs are ligated together. The splicing process occurs in cellular machines called spliceosomes, in which the snRNPs are found along with additional proteins (Figure 1.13).

Alternative splicing, in addition to basic splicing, occurs in over 90% of the human genome conferring great diversity within the human proteome. The different isoforms generated during alternative splicing from a single gene tend to be expressed in a tissue-specific fashion and are regulated by the splicing code. Different elements such as cis and trans acting elements within the splicing code modulate the strength of the splice site and the resulting splice variant (Neil and Fairbrother, 2019). Alternative splice sites can be significantly enhanced by trans-acting elements, RNA binding proteins (RBPs). RBPs can either bind to cis-element, intron splicing enhancer (ISE) or the exon splicing enhancer
(ESE) to enhance splicing. On the other hand, it can also bind to the intron splicing silence (ISS) or exon splicing silencer (ESS) to inhibit spliceosomes binding to the splice site (Black et al., 2019). 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).

1.8.2 HnRNP proteins in T cell functions

Heterogeneous nuclear ribonucleoproteins (hnRNPs) represent a large family of RNA-binding proteins (RBPs) that contribute to multiple aspects of nucleic acid metabolism including alternative splicing, mRNA stabilization, and transcriptional and translational regulation (Geuens et al., 2016). There are 20 major members in the hnRNP protein family. These proteins 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. Four unique RBDs were identified in hnRNP proteins: the RNA recognition motif (RRM), the quasi-RRM, a glycine-rich domain constituting an RGG box and a KH domain (Geuens et al., 2016).

hnRNPL was first implicated in T cell biology through its role in regulating the splicing of the CD45 gene, which encodes a transmembrane phosphatase essential for T cell activation (Rothrock et al., 2005). Rothrock and colleagues have shown previously that hnRNPL is a key determinant of CD45 splicing and expression. 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 (Rothrock et al., 2005). The different variants are expressed in different cell types based on the maturity and activation status of the cells. 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 (Rothrock et al., 2005). Additionally, memory T cells are also characterized by the expression of CD45RO. Once these two isoforms dimerize, 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 two hnRNP proteins: hnRNPL and hnRNPLL. 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 allowing for exon 5 to be included in the lariat and be spliced out of the transcript. HnRNPL can bind to exon 4 but not to exon 5 or 6, thus it is unable to generate the CD45RO isoform alone (Preußner et al., 2012; Figure 1.14). Other functions of hnRNPLL include global splicing regulator of 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).

1.9 The role of Sirtulin-1(Sirt-1) in T cells

1.9.1 Overview of Sirt1 Biology

Sirtuin 1 (Sirt1), encoded by the Sirt1 gene, is the most conserved mammalian
nicotinamide adenine dinucleotide (NAD\(^+\)) dependent histone deacetylase (Salminen et al., 2013). Sirt1 plays a crucial role in metabolism and inflammatory responses (Figure 1.15). Sirt1 functions as a deacetylase that can regulate different transcription factors important for modulating immune responses. In addition to Sirt1’s role in immune modulation and cellular metabolism, it as an oncogene depending on the context specificity (Biason-Lauber et al., 2013).

Sirt1 guards against oxidative stress by activating gene transcription of PGC-1\(\alpha\) via deacetylation, and by regulating transcription of factors such as the nuclear receptor peroxisome proliferator-activated receptor (PPAR), nuclear respiratory factor (NRF), and mitochondrial transcription factor A (TFAM), involved in modulation of biogenesis and mitochondrial function (Ren et al., 2019), and metabolism of glucose and lipids (Rodgers et al., 2005).

Sirt1 protects against oxidative stress via regulation of FOXO protein acetylation, which is involved in antioxidant processes, apoptosis, and cell proliferation (Wong and Woodcock, 2009). By activating FOXO/MsSOD pathway, Sirt1 increases the expression of manganese superoxide dismutase (MnSOD) and catalase, counteracting oxidative stress and promoting damage repair (Gu et al., 2016). Sirt1 also interacts with protein substrates in a variety of signaling pathways such as Wnt and Notch.

**1.9.2 Sirt1’s role in T cell functions and effector cells**

Sirtuins depend on NAD\(^+\) (a critical cofactor of metabolism) for their enzymatic activity, thus any fluctuations in NAD\(^+\) levels related to nutrient availability affects their functions (Anderson et al., 2017). Hence, sirtuins can be regarded as energy sensors that
links environmental signals to cellular metabolic homeostasis. T cell effector functions depend on nutrient availability providing a link between sirtuins and metabolic reprogramming of T cells. Thus, sirtuins are speculated to be potential regulators of the immune response, impacting the ability of the immune system to combat foreign pathogens or malignant cells (Hamidi et al., 2022).

Transcription factor c-Jun is upregulated after T cell activation to induce IL2 production, cell proliferation and differentiation (Atsaves et al., 2019). Zhang and his colleagues indicated in their study that Sirt1 inhibits the immune response by blocking c-Jun transcription factor activity, which supports IL2 production and thereby decreases Th1 cell activation (Zhang et al., 2009). Studies performed by Sequeria in Sirt1 knockout (Sirt1−/−) mice showed that Sirt1−/− T cells displayed increased proliferation and IL2 production, and Sirt1−/− mice are more susceptible to developing autoimmune diseases (Sequeria et al., 2008). Further studies indicated that IL-2 can reverse T cell anergy by suppressing Sirt1 transcription via cytosolic sequestration of its upstream transcription factor, FoxO3a. The expression of a constitutively active form of FoxO3a blocks IL-2-mediated reversal of T cell tolerance by retaining Sirt1 expression (Gao et al., 2012). B cell lymphoma 2-associated factor 1 (Bclaf1), a promoter of cellular apoptosis, has been found to be critical for T cell activation (McPherson et al., 2009). Kong et al. reported that Sirt1 suppresses Bclaf1 activity by deacetylating histone lysine residues at the Bclaf1 promoter region, resulting in decreased IL2 gene transcription. Hence, Sirt1−/− T cells displayed higher expression of the Bclaf1 gene and IL2 production, and specific knockdown of Bclaf1 reversed the increase in IL2 production and proliferation observed in Sirt1−/− T cells (Kong et al., 2011).
The effects of Sirt1 on Th17 cells are less well known, and more studies are needed to understand its precise role in Th17 effector functions. One study showed that, Sirt1 activators such as metformin impede Th17 cell differentiation and reduce IL17A and RORγt expression via deacetylation of the STAT3 transcription factor. STAT3 deacetylation restricts its ability to translocate into the nucleus to induce RORγt transcription (Limagne et al., 2017). Another study showed that in vivo activation of Sirt1 using NAD⁺ supplementation delayed the onset of experimental autoimmune encephalomyelitis (EAE). This protection was hypothesized to be a result of the decrease in Th17-mediated inflammatory responses induced by enhanced Sirt1 expression (Wang et al., 2016). In another study by Wang et al, identified a treatment with methylene blue, another Sirt1 activator, to alleviate Th17 responses and significantly reduce the clinical scores of EAE in mice (Wang et al., 2016). In contrast, other researchers have shown that Sirt1 activation promotes the Th17 phenotype via RORγt deacetylation. Deacetylated RORγt appears to have stronger transcriptional activity than Foxp3, thus strengthening the Th17 proinflammatory phenotype and suggesting that Sirt1 inhibitors may protect against autoimmune diseases (Lim et al., 2015).

Sirt1 has been shown to increase iTreg conversion to Th17 cells by deacetylating the Foxp3 transcription factor in iTregs. Foxp3 is a master regulator of Treg cell development and function and has three lysine acetylation sites (K31, K262, and K267) targeted by Sirt1 (Kwon et al., 2012). Hyperacetylation of Foxp3 prevents its polyubiquitination and proteasomal degradation. Accordingly, Sirt1 deacetylase activity has been found to reduce Foxp3 protein levels, and treatment with Sirt1 inhibitors results
in increased functional Treg cells (van Loosdregt et al., 2010). Some studies reported that genetic deletion or pharmacologic inhibition of Sirt1 increased the number and suppressive activity of Foxp3+ Treg cells by increasing Foxp3 mRNA levels (van Loosdregt et al., 2010). In contrast, Sirt1 was reported to promote Treg cell survival by stabilizing the Notch1 intracellular domain proximal to the membrane, given that the Notch receptor is essential for Treg cell survival within caloric-restricted conditions (Marcel et al., 2017).

1.10 Inflammatory bowel disease (IBD) and treatment modalities

1.10.1 Inflammatory bowel disease

Inflammatory bowel disease (IBD) comprises of disorders involving chronic inflammatory disease of the intestine. The two main pathogenicity of IBD are ulcerative colitis (UC) and Crohn’s disease (CD). IBD is broadly defined by a disruption of immune homeostasis, with aberrant immune responses against gut commensal microbiota. Current incidence of IBD has risen dramatically in recent decades. For instance, in 2015, ~1.3% of US adults (3 million) were estimated to be diagnosed with IBD, representing a 50% increase from 1999 (2 million) amongst all race and ethnic groups (Centers for Disease Control and Prevention). In addition, these patients have increased risks of colon cancer; More than 20% of IBD patients eventually develop colon cancer. IBD overall can severely impact patients’ quality of life. Common symptoms of IBD include abdominal pain, vomiting, diarrhea, and rectal bleeding.

IBD is associated with an imbalance of the intestinal microbiota where the immune regulation of the gut is extremely complex. To maintain homeostasis, the gastrointestinal immune system must be able to both maintain tolerance towards food and the commensal
microflora while also being able to mount a rapid response against pathogens. In the healthy state, gut homeostasis is maintained by suppressing excessive immune responses to foreign antigens from gut non-pathogenic microbiota, self-antigens, and food antigens while synchronously mounting an appropriate response to pathogenic microbes. Intestinal epithelial cells (IECs) produce high levels of TGF-b, a cytokine that is important for regulatory T cell differentiation. During infections, gut Treg cells preserve this equilibrium by simultaneously enabling an effective (Teff) anti-pathogenic response but preventing the development of an inflammatory pathology (de Mattos et al., 2015). Tregs maintains this homeostasis by discriminating between harmless (e.g., dietary compounds and intestinal microbes) and harmful stimuli (e.g., pathogens). Thus, overall toleration of food molecules and microbial components emerges from the ability of Treg cells to establish this crosstalk with the gastrointestinal environment. Failure to coordinate this equilibrium can lead to chronic IBD diseases (de Mattos et al., 2015). Hence, Tregs can be regarded as the “master regulators” of intestinal/immune homeostasis. IBD patients also produce high levels of the pro-inflammatory cytokine, IL6, which can act with TGFβ to promote the differentiation of Th17 cells. IBD patients have more inflammatory Th17 cells and fewer regulatory T cells.

Currently, there is no cure for IBD. Prevailing IBD treatments aim to reduce the destructive pro-inflammatory immune responses that characterize IBD. Current therapy includes the use of anti-inflammatory drugs (i.e., thiopurines, steroids) and immunosuppressants (anti-integrin therapies, anti-receptor antibodies like anti-TNFα). However, while not all patients respond to treatment (e.g., ~30% of IBD patients do not respond to anti-TNFα agents) (de Mattos et al., 2015). Overall, all patients are subjected
to non-specific activity that can result in serious side-effects including increased infection risk, bone marrow dysfunction, organ dysfunction, and increased risk of malignancy. Moreover, even when patients respond initially to current treatments, these treatments frequently fail to maintain prolonged remissions requiring treatment course alterations, additional hospitalization, or surgery to manage complications. As a result, there is a serious clinical need for newer, safer treatments of IBD that address the underlying causes and not just the symptoms.

1.10.2 iTreg therapeutic potential and role in IBD

Several studies have identified Treg deficiency as an important contributor to IBD (Yamada et al., 2016). Yamada et al, showed that the adoptive transfer of Tregs helped prevent and treat experimentally induced colitis. Yamada et al, also showed that iTregs were functionally advantageous over nTregs in established colitis. A recent study demonstrated that antigen specific, ovalbumin reactive Tregs could be infused to treat refractory Crohn’s Disease (Himmel et al., 2012). Several important phenotypical markers from gut Tregs related to suppressive activity have been identified. Among them, CD39 and CD73 are particularly relevant to IBD since ATP from host cell and the microbiota support the development of inflammatory Th17 cells (Himmel et al., 2012). Foxp3+ Tregs can convert into Th17 contributing to disease progression. CTLA4, IL35, IL10, or LAG3 have all been associated with non-suppressive Tregs (Himmel et al., 2012). Reduced iTreg number are frequently found in the Lamina Propria (LP) of IBD patients and those that are present often show reduced suppressive capacity, typically from destabilized expression of Foxp3 and subsequent conversion of iTregs into pro-inflammatory Th1 or Th17 effector cells (Himmel et al., 2012, Figure 1.16).
Cell therapy has been considered as a paradigm shift of modern medicine. However, before significant progress can be made in this area, major investments need to be focused on our understanding of cell-based therapies. One aspect of cell therapy relates to the development and use of Tregs, a subset of CD4 T cells responsible for suppressing activation, proliferation, and effector responses, both of innate and adaptive immune cells. Tregs provide essential immunosuppressive function and are necessary for transplantation tolerance. Moreover, nTregs can be removed from the patient and expanded ex vivo, facilitating the development of a potential ‘therapeutic product’. Although thymically derived nTregs are CD4+CD25+Foxp3+, there are additional markers that impact Treg function and in vivo stability including CD45 isoform expression (CD45RA, -RB, and -RO), expression of Foxp3, CTLA4 amongst others. One of the biggest hurdles with Treg therapy is to stabilize the Treg phenotype in vivo after vigorous ex vivo expansion of these cells. Several studies have determined that the Treg numbers after weeks of transfusions are consistent. However, despite the absolute Treg numbers presented, these Tregs are not suppressive developing 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 highly inflammatory environments.

1.10.3 Intracellular antibody delivery using novel cell-penetrating mimics (CPPMs)

Intracellular antibody delivery offers a new paradigm to understand and control cellular signaling networks. A prominent method for cell therapy in recent years is the
use of antibodies. Antibodies are revolutionizing personal medicine and represent one of the most significant and growing classes of biologics mainly due to their specificity and opportunities to ‘drug’ undruggable targets (Jin et al., 2021). Due to their specificity and tight binding, antibodies provide target design selectivity but without the instability of short nucleic acids. However, current antibody therapeutics limitations include their ability to operate extracellularly, targeting secreted or surface-bound proteins. Intracellular delivery of antibodies is a new concept proven to have great significance, especially into cells like human immune cells, considered ‘hard-to-transfect’ (Jin et al., 2021). In many ways, the success of antibody therapy will depend on the ability to deliver functional forms into living cells. Considerable effort has been focused on intracellular antibody delivery, yet with limited success. Most of these studies utilize immortal cell lines instead of relevant primary human cells. More recently, two additional approaches have been reported that appear promising. Functional proteins were fused to supercharged GFP; however, it appears that intracellular antibody delivery has not yet been attempted. Similarly, ‘cytotransmabs’ showed antibody internalization in HeLa cells. Both approaches require covalent attachment of the active agent to the delivery moiety.

Common method to deliver antibody into cells was using polymeric scaffolds. Polymeric scaffolds were developed with the use of new chemical compositions and polymer architectures, and non-covalent attachment to the cargo. These molecules are called cell penetrating peptide mimics (CPPMs), also known as protein transduction domain mimics (PTDMs), that exhibit highly efficient delivery for biologics, even to hard-to-transfect cells, with higher potency. Recent studies have showed that these
CPPMs have demonstrated greater ability to manipulate immunological responses in the context of T cell engineering (Ozay et al., 2016). The chemical composition of CPPMs have the combinatorial characteristics of TAT (guanidinium-rich), and Pep-1 (sequence of hydrophobic region and cationic domain). The hydrophobic region consists of phenyl-functionalized repeat units, which were shown to be more efficient in cellular penetration with respect to aromatic or aliphatic groups (deRonde and Tew, 2015; Sgolastra et al., 2017).

1.11 Rationale, central hypothesis, specific aims, and significance

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. 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 and induce the production of cytokines that are most efficacious at targeting the specific pathogen.

It was previously 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 T cell type, through a process known as plasticity. Plasticity can occur between several different T cell subsets but is observed most predominantly between iTreg and Th17 cells (Zhou et al., 2009). Th17 and Treg cells regulate the differentiation of one another to maintain equilibrium. Both T cell
lineages require TGF β for their differentiation. TGF β induces the expression of Foxp3 in naïve T cells, promoting the development of Tregs, but in the presence of IL-6, it inhibits the generation of Tregs while it induces the expression of ROR γ t, thus promoting a Th17 phenotype. Retinoic acid has also been involved in the Treg-Th17 balance; it can induce Foxp3 and inhibit ROR γ t even in Th17-inducing conditions, then promoting the development of Tregs.

In some autoimmune diseases, such as in rheumatoid arthritis and inflammatory bowel disease, 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. 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, understanding the molecular mechanisms of how these signals are integrated and how they affect immunological tolerance remains unanswered. Protein kinase Cθ (PKCθ) is a T-cell specific kinase that functions in T cell activation and induction of effector function and is an emerging regulator of the balance between effector T cells and regulatory T cells. 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).

Energy usage differs between T cell subsets. Naïve CD4 T cells are
metabolically quiescent and rely on oxidative phosphorylation as their primary means of generating energy (Chapman et al., 2019). Following activation, effector T cells undergo metabolic changes to accommodate the increased energy demands to meet the proliferation effector functions. Effector T cells use glucose as their primary energy source. In contrast, the glucose transporter 1 is absent in Tregs and Tregs use fatty-acid oxidation (FAO) as their main energy source. Thus, the question remains, how changes in the metabolic profiles may affect the plasticity of different cell subsets represents another area of inquiry.

Recent studies by 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 serine/threonine kinase that mediates key processes including cellular energetics and growth. LKB1 has been shown to protect Treg function by maintaining metabolism, cellular survival, and Foxp3 expression. This suggests that LKB1 regulates phenotypic stability. However, to our knowledge how LKB1 may contribute to Treg-Th17 cell plasticity has not been explored. Identifying key molecules that regulates iTregs and Th17 cells plasticity will aid in developing therapeutics to specifically modulate the ratio of effector to Treg cells. To this end, the present study may also have implications for improving Treg therapies to treat autoimmune diseases. In this study, our central hypothesis is that LKB1 regulates the plasticity axis between Th17 cells and iTreg. Furthermore, it has been shown that IL-6 and PKCθ activity can both destabilize the Treg phenotype. We will test these hypotheses through the specific
aims outlined in the next chapters.
Figure 1.1: Innate and adaptive immunity timeline. The mechanisms of innate immunity provide the initial defense against infections. Adaptive immune responses develop later and require the activation of lymphocytes. The kinetics of the innate and adaptive immune responses are approximations and may vary in different infections. Adapted from Cellular and Molecular Immunology, 8th edition, Figure 1-1.
Figure 1.2: A graph showing the difference in timing between the innate and adaptive immune response and amplitudes in primary and secondary response to antigen. The primary adaptive immune response to antigen is low in both duration and amplitude, while subsequent exposure to the same antigen results in secondary responses. These secondary responses recruit memory cells and are thus faster to appear, stronger in amplitude, and more specific to the antigen than was the primary response (dark blue). In contrast, innate immune responses, which do not form immunological memory, remain unchanged every time the antigen is encountered (light blue). Adapted from Cellular and Molecular Immunology, 8th edition, Figure 1-8.
Figure 1.3: T cell selection in the thymus. T cell progenitors migrate from the bone marrow to the thymus, where they become thymocytes and complete their development by rearranging their T cell receptor genes and undergoing repertoire selection. (a & b) During the process of positive selection, immature T cells that can bind self-MHC receive survival signals, while those which fail to recognize self-MHC are eliminated through the induction of apoptosis. (c) Next, the process of negative selection selects self-tolerant thymocytes by providing survival signals, whereas those which react too strongly with self-antigen are deleted from the repertoire by apoptosis.
Figure 1.4: 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 1.5: A schematic of JAK/STAT signaling. Cytokines bind a specific receptor to illicit a cellular response. JAK kinases are bound to the cytokine receptor and become auto phosphorylated 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 1.6: 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 1.7: Energy usage differs between T cell subsets. There are differences in energy usage between different T cell subsets. Naïve T cells use glucose, fatty acids, and amino acids as their energy source. Effector T cells have higher energy efficiency and use glucose as their primary energy source. In contrast, the glucose transporter 1 is absent in Tregs and Tregs use fatty-acid oxidation (FAO) as their main energy source. Adapted from Galgani et al., 2015.
Figure 1.8: 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 1.9: iTreg and Th17 cell phenotypes are plastic.** Th17 and Treg cells regulate the differentiation of one another to maintain equilibrium. Both lineages T requires TGFβ for their differentiation. TGFβ induces the expression of Foxp3 in naive T cells, promoting the development of Tregs, but in the presence of IL6, it inhibits the generation of Tregs while it induces the expression of RORγt, thus promoting a Th17 phenotype. In addition, TGFβ induced Foxp3 expression negatively regulates Th17 cell differentiation by repressing RORγt expression.
Figure 1.10: The main isoforms of Stk11. Stk11 is the gene that encodes 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 1.11: PKCθ is phosphorylated upon TCR stimulation. Protein kinase C θ (PKCθ) is a T cell specific kinase that functions in T cell activation and induction of effector function. 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. Adapted from Sutcliffe et al., 2011.
Figure 1.12: PKCθ promotes Th17 differentiation and destabilizes iTreg function. Studies have shown that PKCθ is crucial for Th17 cell differentiation as PKCθ−/− T cells exhibit a diminished Th17 phenotype. In Tregs, PKCθ has been shown to have an inhibitory role especially to their suppressive activity.
Figure 1.13: 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 1.14: 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 Preußner et al., 2012.
Figure 1.15: NAD$^+$-dependent Sirt1 deacetylase reaction. Sirt1 is dependent on NAD$^+$ for catalysis. Sirt1 deacetylates target proteins by hydrolyzing NAD$^+$ and simultaneously transferring the lysine-bound acetyl group from acetylated proteins to the 2′-OH position of ADP-ribose, ultimately yielding nicotinamide and 2′-O-acetyl-ADP-ribose. Adapted from Yang et al., 2022.
Figure 1.16: Mechanisms of Treg cell-mediated suppression. Treg cells use a multitude of mechanisms to promote a tolerogenic microenvironment and tissue repair. (A) Secretion of the anti-inflammatory cytokines, IL10, TGFβ and IL35, not only inhibit Teff cell proliferation but also suppress Th1 and Th17 effector cell function, which are key mediators of IBD. (B) Treg cells express the high-affinity IL-2 receptor α-chain (CD25) consuming local IL2 with greater affinity than effector cells. (C) Tregs co-expressing CD39 and CD73 disrupt metabolic processes in effector cells by converting ATP into pericellular adenosine, a potent inhibitor of Teff cell function. Additionally, adenosine stimulates TGFβ production, promoting development of pTregs. (D) Tregs are capable of secreting perforin, granzyme B, and galectin-1 which are directly cytotoxic to Teff cells. Activated Treg cells also express TRAIL, inducing apoptosis of Teff cells through the TRAIL/DR5 pathway. (E) Expression of CTLA4 degrades DC-derived CD80 and CD86 leading to impaired CD28-mediated co-stimulation of T cells. DC function is further inhibited through the interaction of Treg-derived TIGIT and CD155 on DCs. This induces IL10 production and suppresses IL12. (F) In response to alarmins, Treg cells produce AREG, an important regulator of tissue repair and regeneration. AREG, amphiregulin; CTLA4, cytotoxic T lymphocyte associated protein 4; DC, dendritic cell; DR5, death receptor 5; IL, interleukin; pTregs, peripheral regulatory T cells; Teff, T effector lymphocyte; TGFβ, transforming growth factor beta; Th1, T helper type 1 cell; Th17, T helper type 17 cell; TIGIT, T cell immunoreceptor with Ig and immunoreceptor tyrosine-based inhibitory motif domains; TRAIL, TNF-related apoptosis-inducing ligand; Treg, regulatory T cell. Adapted from Clough et al., 2020.
CHAPTER 2

LKB1 isoform expression modulates T cell plasticity downstream of PKCθ and IL-6

2.1 Introduction

Naïve CD4 T cells are activated by antigen-specific signals conveyed through the T cell receptor (TCR) and co-stimulatory signals, transmitted through CD28. A third signal, relayed by cytokines, direct activated T cells toward a specific differentiated helper phenotype. It was originally thought that T cell differentiation was terminal and could not be changed (Zhou et al., 2009). Over the past decade, evidence of T cell plasticity has emerged. This has become an important research focus, especially in the context of autoimmune conditions, in which a diverse milieu of proinflammatory cytokines can induce pleiotropic effects.

Autoimmunity is characterized by the failure of immunological tolerance to inhibit aberrant immune response to self-tissue (Michalek et al., 2011, Ren, Li, 2017). Regulatory T cells (Tregs) maintain tolerance by preventing or terminating effector T cell activity. Naturally occurring Tregs (nTregs) are generated in the thymus while, in the periphery, naïve CD4 T cells can differentiate into induced regulatory T cells (iTregs) in the presence of Transforming growth factor β (TGFβ) and Interleukin-2 (IL-2) (Zhou et al., 2009, Priyadharshini et al., 2018). In the case of autoimmunity, Treg function is significantly compromised. There is speculation as to whether this diminished functionality is due to plasticity – the ability of Tregs to adopt phenotypic and functional aspects of proinflammatory T helper (Th) cells. This phenomenon, especially involving Treg and Th17 cells, has been well-documented to occur both in vitro and in vivo (Gerriets et al., 2018, Zhou et al., 2009, Almeida et al., 2016, Galgani et al., 2015, Mucida et al., 2007).
In highly inflammatory environments, such as in colitis and rheumatoid arthritis, populations of Tregs have been identified that express the Th17 master transcription factor, RAR-related orphan receptor gamma (ROR\(\gamma\)). These ROR\(\gamma\)Tregs can produce and secrete the signature Th17 cell cytokine, Interleukin-17 (IL-17) (Ren, Li, 2017). Furthermore, both in vitro and in vivo studies showed that Th17 cells can transdiffernetiate into forkhead box P3 (Foxp3+) T cells, in response to certain conditions, and this reprogramming improved disease survival in mouse-models of Th17-mediated autoimmunity (Mucida et al., 2007, Gagliani et al., 2015, Gualdoni et al., 2016, Wang et al., 2016, Sun et al., 2017).

Protein kinase C theta (PKC\(\theta\)) is a PKC family member most abundant in T cells, where it performs critical functions in effector T (Teff) cell activation. PKC\(\theta\) uniquely localizes to the center of the T cell immunological synapse (IS) through its physical association with CD28. This localization is required for downstream PKC\(\theta\)-mediated signaling. Studies have demonstrated that purified PKC\(\theta\)−/− T cells exhibit significantly diminished proliferation and IL-2 production due to defects in activator protein 1 (AP-1), nuclear factor kappa (NF-\(\kappa\)B), and nuclear factor of activated T cells (NFAT) pathways. This has been confirmed by multiple studies using strains of independently generated PKC\(\theta\)-deficient mice (Sun et al., 2000, Manicassamy et al., 2006, Altman et al., 2004). PKC\(\theta\) has opposing functions in effector and regulatory T cells. PKC\(\theta\) is crucial for Th17 cell differentiation and PKC\(\theta\)−/− T cells are deficient in their ability to adopt a Th17 phenotype.

There are several Th17 differentiation mechanisms that require PKC\(\theta\) function. Interleukin-6 (IL-6) signaling is necessary for Th17 differentiation. Downstream of IL-6,
signal transducer and activator of transcription 3 (STAT3) is activated and induces the upregulation of RORγt and IL-17, and PKCθ regulates STAT3 expression (Kwon et al., 2012). PKCθ activates sterol regulatory complex 1 (SRC1), which regulates the transcription of Rorc, the gene encoding RORγt (Sen et al., 2018). As such, PKCθ is crucial for Th17 cell differentiation and effector function through its modulation of IL-17 production. In Tregs, PKCθ has been shown to have inhibitory effects. 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 forkhead box protein O1/3a (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 (TNFα) production, which acts to prevent Tregs from undergoing TNFα-mediated inactivation (Zanin-Zhorov et al., 2012). In autoimmune models, inhibiting PKCθ in Treg cells enhances their suppressive capacity in a proinflammatory autoimmune environment (Zanin-Zhorov et al., 2012). Altogether, these studies suggest an important role for PKCθ in fine-tuning the balance between Teff and Treg responses.

Alternative splicing occurs in over 90% of the human genome, which confers great diversity within the human proteome. 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. Alternative splice sites are usually weak but can be significantly enhanced by trans-
acting elements, such as RNA binding proteins (RBPs) that include heterogenous nuclear ribonucleoproteins (hnRNP) (Béthune et al., 2019).

HnRNPL-like (hnRNPLL) is one of 20 major members of the hnRNP family. The canonical example of hnRNPLL-mediated splicing effects on T cell activation is found in the CD45 isoforms, CD45RA, -RB, and -RO (Lynch, 2004, Holmes et al., 2006, Miosge et al., 2007, Oberdoerffer et al., 2008). HnRNPLL is upregulated following CD28 engagement and mediates Prprc (CD45) splicing to control T cell activation (Butte et al., 2012). HnRNPLL expression and the number of spliced transcripts are both increased following CD28 engagement. Additional gene targets of hnRNPLL that could impact T cell differentiation and function include CD44, signal transducer and activator of transcription factor 5a (STAT5a), and fatty acid synthase.

Liver kinase B1 (LKB1), is a serine/threonine kinase that mediates key processes including cellular energetics and growth. Encoded by Stk11, it was first identified as a tumor suppressor that, when mutated, causes spontaneous tumors such as those found in Peutz-Jeghers syndrome (Blagih et al., 2012). LKB1 functions in energy-sensing by activating 5’ adenosine monophosphate protein kinase (AMPK) to further initiate fatty acid oxidation by inhibiting mammalian target of rapamycin (mTORC1), and activation of the fatty acid oxidation enzyme carnitine palmitoyltransferase 1a (CPT1a; Lochner et al., 2015, Ma et al., 2017). Several groups have shown severe autoimmunity develops in mice with LKB1 conditionally deleted from Tregs. LKB1 has been shown to protect Treg function by maintaining metabolism, cellular survival, and Foxp3 expression (He et al., 2017, Wu et al., 2017, Yang et al., 2017). Differential expression of LKB1 isoforms has been described, and Stk11 splice variants have been characterized in healthy and diseased tissues.
(Kong et al., 2017, Denison et al., 2009, Donnelly et al., 2021). To our knowledge, no study has explored how LKB1 may contribute to Treg-Th17 plasticity.

This study describes a mechanism whereby changes in LKB1 isoform expression, generated when Stk11 is alternatively spliced, facilitate phenotypic plasticity between iTreg and Th17 cells, in the context of IL-6 and PKCθ signaling. Defining the mechanisms that act as the nexus of iTreg-Th17 cell plasticity has the potential to significantly advance our understanding of this phenomenon and aid the development of stable cell-based therapies to treat aberrant immune-mediate conditions.

2.2 RESULTS

2.2.1 The short isoform of LKB1 is predominantly expressed in Th17 cells

LKB1 is an essential regulator of fatty acid oxidation and has been shown to regulate Treg metabolism (He et al., 2017). It was hypothesized that iTregs, which mainly utilize fatty acid oxidation for energy, would express more LKB1 compared to Th17 cells that are highly glycolytic. We stimulated naive splenic CD4 T cells and cultured them under Th17, iTreg, or non-polarizing (NP) conditions for a period of 7 days (Figure S1A), and assessed LKB1 expression using flow cytometry. Greater than 90% of Th17 and iTreg cells expressed LKB1 (Figure 2.1A). Th17 cells expressed approximately three times more LKB1 than did iTreg or NP cells, as measured by median fluorescence intensity (MFI; Figure 2.1B). We confirmed by immunoblot that LKB1s is more abundantly expressed in Th17 cells than in iTregs (Figure 2.1C).

LKB1 can exist as either a short (LKB1S) or a long (LKB1L) isoform. It is encoded by the gene, Stk11, and 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 (Denison et
al., 2009). Exon 9a contains a stop codon, leading to a truncated transcript with no 3’UTR (Figure S1B). Exon 9a is considered a cryptic exon that is alternatively spliced from intron 8 (Figure S1C). Based on the unexpectedly high level of LKB1 detected in Th17 cells, we revised our hypothesis, and asked whether Th17 and iTreg cells express different isoforms of LKB1. We designed primers to specifically amplify exon 9a in Stk11s, exon 9b in Stk11l, as well as to exon 1, which is expressed in both Stk11s and Stk11l, and referred to as the “common” exon (Figure S1D). Using qRT-PCR, we determined that Th17 cells express approximately 2-fold more Stk11s compared to iTregs (Figure 2.1D). Conversely, iTregs express greater than two-fold more Stk11l compared to Th17 cells (Figure 2.1E). The ratio of Stk11s:Stk11l expression was significantly higher in Th17 cells compared to iTregs (Figure 2.1F). Our data shows that Th17 cells express higher levels of LKB1 than do iTregs. Furthermore, Th17 and iTreg cells express different splice variants of Stk11, with Th17 cells expressing significantly more Stk11s/LKB1s, and iTregs expressing predominantly Stk11l/LKB1l.

2.2.2 HnRNPLL associates with Stk11s in Th17 cells and requires PKCθ for its expression

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 RBP-transcript association by identifying binding motifs within the transcript sequence (Paz et al., 2014). We analyzed the Stk11 sequence using RBPmap scan and identified the heterogenous nuclear ribonucleotide protein L-like (hnRNPLL) as one of the RBPs that showed strong binding potential. HnRNPLL has been shown to be important for
generating CD45 isoforms in T cells (Wu et al., 2008). Therefore, we asked whether hnRNPLL might also function in Stk11 splicing.

Measuring the expression of hnRNPLL in Th17 and iTreg cells found that Th17 cells express more hnRNPLL than do iTregs at the protein and the transcript levels (Figures 2.2A, 2.2B, respectively). We asked whether differences in hnRNPLL expression have functional consequences for Stk11 processing in Th17 and iTreg cells and generated whole cell lysates from Th17 and iTreg cells, in which RBPs were bound to RNA by a cross-linking step. We immunoprecipitated hnRNPLL from the cell lysates to enrich for RNA transcripts that were bound to hnRNPLL, de-crosslinked the samples, and extracted the RNA. Stk11s primer set was used to determine whether Stk11s preferentially associated with hnRNPLL in Th17 cells, as compared to iTregs. As shown in Figure 2.2C, significantly more Stk11s transcript bound to hnRNPLL in Th17 cells, than bound to iTregs. The collective data indicates that hnRNPLL is more highly expressed in Th17 cells and is found preferentially bound to Stk11s in Th17 cells, compared to iTreg cells.

2.2.3 PKCθ regulates Stk11 splice variant and LKB1 isoform expression upstream of hnRNPLL

Protein Kinase Cθ functions downstream of CD28 signaling. It is required for Th17 differentiation, while also acting to inhibit iTreg function (Altman et al., 2004, Sen et al., 2018, Wu et al., 2008). PKCθ has also been shown to regulate splicing activity through splicing factors, such as SC-35 (Sen et al., 2018). Therefore, we inquired as to whether PKCθ regulates Stk11 splicing to generate LKB1s in Th17 cells. Compared to iTregs, there was significantly more Prkcq, the transcript that encodes PKCθ, in Th17 cells (Figure 2.3A).
To determine whether PKCθ regulates Stk11 splicing, we first examined hnRNPLL expression in WT and PKCθ−/− Th17 cells (Figure 2.3B). In WT Th17 cells that express PKCθ, robust levels of HnrnpII were observed, whereas in Th17 cells lacking PKCθ, HnrnpII expression was significantly diminished. This confirmed PKCθ is necessary for HnrnpII expression in Th17 cells (Figure 2.3B). With this observation, we hypothesized that within PKCθ−/− Th17 cells would be defective Stk11 splicing. It was found that compared to WT Th17 cells, there is significantly lower expression of Stk11S and LKB1S in Th17 cells without PKCθ (Figures 2.3C, D, respectively). These data support a model whereby PKCθ regulates hnRNPLL expression in Th17 cells, which itself mediates Stk11 alternative splicing.

2.2.4 HnRNPLL regulates Stk11S expression

It was previously demonstrated that restricting the function of an intracellular target protein through the use of a specific antibody complexed to a synthetic peptide containing a protein transduction domain mimic (PTDM) transports the target across the cell membrane (Kong et al., 2017). To confirm how hnRNPLL regulates Stk11 splicing, siRNA was used to knock down hnRNPLL in WT Th17 cells. We cultured CD4 T cells from WT or PKCθ−/− mice under Th17 polarizing conditions. On day 3 of the differentiation process, cells were transfected with either an siRNA to hnRNPLL (siLL) or a scrambled siRNA as a negative control (siScr). RT-qPCR and immunoblotting were used to confirm our knockdown was successful at both the transcript and the protein level (Figure S2A, B). Compared to Th17 cells, or to Th17 cells transfected with s-Scr, Th17 cells in which HnrnpII was knocked down (Th17+siLL) showed significantly less Stk11S, and significantly more Stk11L (Figure 2.4A, B). Immunoblotting confirmed at the protein level, that LKB1S expression is
significantly reduced in Th17+siLL cells compared to Th17 or Th17+Scr cells (Figure 2.4C). Th17+siLL cells also showed defective Th17 differentiation, as characterized by diminished \textit{Il17f}, which encodes the Th17 effector cytokine, IL-17 (Figure 2.4D). We observed significantly less \textit{Prkcq} in Th17+siLL cells, (Figure 2.4E). These data confirm hnRNPLL is necessary to induce \textit{Stk11} expression in Th17 cells, and further point to a potential self-reinforcing signaling loop, whereby of PKC\(\theta\) promotes hnRNPLL upregulation, and \textit{Hnrnpl}l is necessary to maintain \textit{Prkcq} expression.

Treg cells function to suppress the activation of nearby T cells, through mechanisms that are both direct, (i.e., cell-cell contact), and indirect, (i.e., release of anti-inflammatory cytokines). The study aimed to determine if hnRNPLL-\textit{Stk11} splicing may regulate Th17 cell \textit{versus} iTreg identity. Treg cells are defined by the CD4\(^{+}\)CD25\(^{hi}\)FOXP3\(^{hi}\) phenotype and possess potent suppressive capabilities. However, iTreg phenotype alone does not demonstrate functional suppression. A standard \textit{in vitro} suppression assay was used to determine whether Th17+siLL cells exhibited suppressive activity that was more comparable to iTreg cells, than to their Th17 counterparts. CD4 T cells were activated to mimic physiological conditions by stimulating them with soluble anti-CD3 and anti-CD28. These responder (T\(_{res}\)) T cells were labeled with the vital dye UltraGreen and mixed in culture at three different ratios with \textit{ex vivo}-differentiated suppressor iTreg (T\(_{sup}\)) cells. A second vital dye, Red650, was used, which emits fluorescence at a longer wavelength, to label the T\(_{sup}\) cells. We used flow cytometry to track the proliferative responses of T\(_{res}\) cells and T\(_{sup}\) cells at the end of the 3-day suppression assay. When Th17+siLL was cultured with T\(_{res}\) cells at a 1:10 ratio, the suppression observed was as strong as when iTreg cells were mixed with T\(_{res}\) cells at a 1:1 ratio. This suggests that Th17+siLL potently suppress T
cell proliferation in standard suppression assays (Figure S2C). The altered, more iTreg-like phenotype, accompanied by increased suppressive activity, was observed in WT Th17+siLL but not in PKCθ−/− Th17+siLL cells. This was likely because of the low endogenous levels of hnRNPLL found in these cells (Figure S2A). Altogether, these data demonstrate that hnRNPLL is critically important for mediating Stk11 alternative splicing in Th17 cells and that PKCθ is required for its expression. In Th17 cells with reduced levels of hnRNPLL, Stk11S levels decrease, Stk11L expression increases, and these cells adopt an iTreg-like phenotype with enhanced suppressive activity.

2.2.5 IL-6 signaling acts upstream of PKCθ-regulated Stk11 splicing

Pro-inflammatory cytokine, IL-6, promotes Th17 cell differentiation in vitro and in vivo. IL-6 receptor-deficient mice are unable to generate Th17 cells (McCauig et al., 2015, Morishima et al., 2009). IL-6 signals through Janus Kinase (JAK) and signal transducers and activators of transcription (STAT) 3 to regulate Rorc, the gene encoding Th17 master transcriptional regulator, RORγt. In some in vivo models of colitis, which are accompanied by high levels of IL-6 expression, FOXP3+ Treg cells can upregulate Rorc and lose their suppressive activity. This suggests that IL-6 is a key regulator of plasticity between iTreg and Th17 phenotypes (Ren, Li, 2017). Conversely, Th17 cells can transdifferentiate into RORγtloFOXP3*IL-10-expressing cells in vivo, further highlighting their plasticity these two phenotypes display (Gaglani et al., 2015). HnRNPLL is induced following signaling through CD28 (Butte et al., 2015), but its expression downstream of IL-6 signaling has not been investigated.
To probe the mechanism by which IL-6 destabilizes iTreg cells, CD4 T cells were polarized under iTreg conditions and were dosed with IL-6 on day 5 of the differentiation process. Fully differentiated iTreg cells that were exposed to IL-6 significantly upregulated \textit{Prkcq} and \textit{Hnrnpll} expression (Figures 2.5A, B, respectively), raising the intriguing possibility that this might also lead to changes in \textit{Stk11} splicing. In differentiated iTreg cells cultured with IL-6, we observed significant increases in \textit{Stk11s} and \textit{Rorc} expression (Figures 2.5C, D, respectively). Thus demonstrating IL-6 exposure not only alters \textit{Stk11} splicing, but also promotes the acquisition of a Th17-like phenotype in iTreg cells.

We questioned whether changes in \textit{Stk11s} expression impacted suppressive capacity, this time in the iTregs in which \textit{Stk11s} was upregulated. To determine whether iTregs+IL6 cells exhibited altered suppressive activity compared to their iTreg counterparts cultured without IL-6, we utilized a standard suppression assay, as before. When iTregs+IL6 T\textsubscript{sup} cells were cultured with T\textsubscript{res} cells at a 1:10 ratio, the suppression observed was significantly lower compared to iTreg cells differentiated without IL-6 (Figure 2.5E). A similar decrease in suppressive capacity in PKC\(\theta^+\) iTreg cells cultured with IL-6 was not observed, which underscores the requirement for functional PKC\(\theta\) for iTreg cells to become Th17-like following IL-6 exposure. Collectively, these data support an outside-in signaling pathway whereby iTreg cells respond to IL-6 by upregulating \textit{Prkcq}, re-expressing hnRNPLL, and generating higher levels of \textit{Stk11s} to facilitate iTreg to Th17 plasticity.

\subsection*{2.2.6 \textit{Stk11} splicing proceeds through a STAT3 mediated pathway}

The T helper (Th) 17 cell program is initiated by IL-6 signaling through the IL-6 receptor (IL-6R) and subsequent activation of Janus Kinase 1 (JAK1) and STAT3 (17, 41).
IL-6R is a Type I transmembrane receptor expressed on immune cells, such as CD4 T cells and monocytes. Following its binding to IL-6, IL-6R associates with a longer transmembrane protein, gp130, which is constitutively bound to a JAK protein. This in turn initiates autophosphorylation of JAK proteins, and subsequent STAT3 activation. In coordination with NF-κB and AP-1, PKCθ stimulates STAT3 expression and STAT3 dimers binds to Rorc and Il17 promoters, inducing the effector functions of Th17 cells.

We were intrigued by our finding that IL-6 and PKCθ regulated hnrNPLL expression and consequently Stk11 splicing. The only previously reported connection between IL-6 and PKCθ was through PKCθ-mediated STAT3 upregulation in Th17 cells (Kwon et al., 2012). It has been shown that IL-6 and PKCθ activity can destabilize the Treg phenotype, although the mechanisms responsible for this have not been fully elucidated. To further define this destabilizing process, CD4 T cells were polarized under Th17 or iTreg conditions and treated with 10 μM Stattic, a specific STAT3 inhibitor, on days 5 and 6 during polarization.

Stattic inhibits STAT3 activation by blocking critical phosphorylation and dimerization events. In some iTreg cell cultures, IL-6 was added on day 5, before adding Stattic (iTreg+IL6; iTreg+IL6+Stattic). Compared to iTreg+IL6 cells, iTreg+IL6+Stattic cells showed significantly lower Prkcq and Hnrnpll expression (Figures 2.6A, B). HnRNPLL was concomitantly reduced in iTreg+IL6+Stattic cells compared to iTreg+IL6 cells (Figure S3A). As expected, inhibiting STAT3 function significantly decreased IL-17 levels, while increasing FOXP3 expression in iTreg+IL6+Stattic cells, when compared to iTreg+IL6 cells (Figures 2.6C-F).
Reduced IL-17 expression also correlated with reduced \textit{Il17f} and Rorγt expression in iTreg+IL6+Stattic cells (Figure S3B-D). At the protein level, LKB1s isoform expression was diminished and LKB1L expression was higher in iTreg+IL6+Stattic cells, compared to iTreg+IL6 cells with intact STAT3 activity (Figure S3E). Altogether, these data provide evidence that IL-6 signals through STAT3 to upregulate \textit{Prkcq} and \textit{Hnrnpll}, modulate \textit{Stk11} alternative splicing, and destabilize the iTreg phenotype, promoting iTreg to Th17 cell plasticity.

\textbf{2.2.7 \textit{Stk11S} expression is sufficient to induce Th17 programming}

Data collected in this study indicates that Th17 cells express higher levels of LKB1 than do iTregs (Figure 2.1B), and Th17 and iTreg cells express different splice variants of \textit{Stk11}, with Th17 cells expressing significantly more \textit{Stk11S} and LKB1S compared to iTreg cells (Figures 2.1C, F). To confirm a direct contribution of the differential \textit{Stk11} splice variants to iTreg and Th17 cell differentiation, it was postulated whether modulating endogenous levels of \textit{Stk11S} in iTreg cells or of \textit{Stk11L} in Th17 cells would influence T cell differentiation during the polarization process. In essence, iTreg cells redirected to adopt a Th17-like phenotype by overexpressing \textit{Stk11S}, and in Th17 cells, overexpressing \textit{Stk11L} to behave more like iTreg cells.

To do this, RNA from CD4 T cells were isolated, reverse-transcribed \textit{Stk11S} or \textit{Stk11L} cDNA, and cloned into pMRX-IRES-GFP retroviral vectors. We used an empty pMRX-IRES-GFP vector (EV) as a negative control. Using immunoblotting, a decrease of LKB1S in the Th17+\textit{Stk11L}-transfected cells compared to Th17 or Th17+EV cells (Figure 2.7A, S3A) was observed. Conversely, iTreg+\textit{Stk11S}-expressing cells had greater amounts
of LKB1s than did iTreg or iTreg+EV cells (Figure 7A). Similar trends in Stk11 expression were also observed (Figure S4A, B).

We quantified Foxp3, Rorc, and Il17f levels in Stk11-transfected cells and found that Foxp3 transcripts were significantly higher and Il17f and Rorc were significantly lower in the Th17+Stk11L cells, compared to Th17 or Th17+EV cells (Figure 7B-D). Foxp3 was significantly higher in iTreg+Stk11s-expressing cells, compared to iTreg cells or iTreg cells transfected with EV, while Il17f and Rorc were expressed at significantly lower levels (Figures 2.7B-D). These results were seen in both WT and PKCθ-/- cells, although the magnitude of the differences was greater in WT cells. These findings provide compelling evidence that Stk11 splice variants act downstream of PKCθ and are both necessary and sufficient to modulate iTreg to Th17 cell plasticity.

2.3 Discussion

Plasticity between T helper cell subsets, such as Tregs and Th17 cells, has been observed in various autoimmune conditions (Ren, Li, 2017). In response to TGFβ signaling, CD4 T cells will upregulate FOXP3 and RORγt, master transcriptional regulators of Treg and Th17 cell differentiation, respectively. However, when TGFβ signals in the presence of the proinflammatory cytokine IL-6, the Foxp3 gene locus is deacetylated and Foxp3 protein is degraded, allowing induction of the Th17 program. It has been well-documented that in the presence of high IL-6, Tregs adopt a Th17-like phenotype (Ren, Li, 2017, Priyadharshini et al., 2018). The mechanisms by which Treg cells assume a Th17-like phenotype, in the context of IL-6 signaling, have remained elusive.
The T cell specific kinase, PKCθ, appears to function at the nexus of Treg-Th17 cell fate choice, preventing the induction of iTreg programming while promoting Th17 cell differentiation. One means by which PKCθ inhibits nTreg, but not iTreg cell function, is by inactivating TNF signaling (Zanin-Zhorov et al., 2010). Conversely, PKCθ is required for Th17 differentiation in vitro, and serves to stabilize RORγt (Sen et al., 2018). PKCθ acts in various capacities in mature CD4 T cells, such as regulating the components of cellular splicing complexes, SC-35, to facilitating NF-κB activation (McCuaig et al., 2015, Schaper et al., 2015).

In this study, we demonstrate PKCθ is required for hnRNPLL expression, as in PKCθ−/− Th17 cells, Hnrnpll expression is severely diminished. This finding further implicates PKCθ signaling in regulating alternative splicing machinery. PKCθ interacts with 14-3-3ζ and NF-κB to form a transcriptional complex in the promoter region of various genes to drive cytokine production, including IFNγ and IL-17 (Schaper et al., 2015). Interestingly, CD28 co-stimulation, which induces PKCθ phosphorylation and its recruitment to the immunological synapse, also upregulates hnRNPLL expression and function (Zanin-Zhorov et al., 2010). Our data are consistent with a model whereby hnRNPLL expression requires intact PKCθ signaling and is reduced in cells that lack PKCθ.

Importantly, this study demonstrates for the first time that IL-6 signaling acts upstream of and positively regulates PKCθ by increasing Prkcq transcription. Additional evidence provides that PKCθ, in turn, regulates the expression of hnRNPLL and subsequent Stk11 splicing. Previous studies demonstrated PKCθ acts to inhibit Treg differentiation and function (Zanin-Zhorov et al., 2010).
2010, Shin et al., 2014). The data presented in this study links IL-6 to \textit{Prkcq} regulation, thus filling a critical gap in our understanding of how IL-6 signaling acts to destabilize the Treg phenotype through PKC\(\theta\).

LKB1 is emerging as an important regulator of Treg function and survival through mechanisms that act through STAT-, \(\beta\)-catenin-, and microtubule-affinity-regulating kinase signaling (Sen et al., 2018, Ma et al., 2012, Zanin-Zhorov et al., 2010). These are in addition to AMPK-dependent LKB1 activities, which facilitate fatty acid oxidation and autophagic processes in Tregs. However, whether LKB1 functions in Th17 cell differentiation has not been explored previously. Our data identified a significant increase in LKB1 expression in Th17 \textit{versus} iTreg cells, when CD4 T cells were polarized \textit{in vitro} (Figure 1). It was further determined that the gene encoding LKB1, \textit{Stk11}, is alternatively processed to generate the \textit{Stk11S} short splice variant, which is expressed abundantly and primarily in Th17 cells (Figure 3). As this study sought to define the regulatory mechanisms responsible for generating the \textit{Stk11} splice variants more precisely, we used RNA immunoprecipitation to demonstrate hnRNPLL is the RNA binding protein that associates predominantly with \textit{Stk11S}, strongly implicating it in \textit{Stk11} splicing. It was also confirmed that functional hnRNPLL is necessary for robust \textit{Stk11S} processing in Th17 cells when siRNA was used to knock-down \textit{Hnrnpll}, \textit{Stk11S} levels decreased significantly (Figure 2).

How LKB1 functions in effector T cells has not been fully elucidated. In peripheral T cells, LKB1 depletion increases glycolysis, cell death, and cytokine production by Th1 and Th17 cells (Shin et al., 2014). Considering recent data demonstrating LKB1 expression in Treg cells is necessary for their integrity (He et al., 2017, Wu et al., 2017, Yang et al.,
2017), the increased cytokine production in LKB1-deficient T cells observed by MacIver et al. (2011), could be the result of functionally defective Treg cells (MacIver et al., 2011). Finding high LKB1 expression and specifically, elevated levels of LKB1s in Th17 cells was surprising. In fact, we determined that LKB1s is expressed preferentially in Th17 cells (Figure 1). It is possible that preferential LKB1s expression may act to maintain glycolytic and fatty acid synthesis processes in Th17 cells, although additional experiments are needed to confirm this. What we can conclude, is that IL-6 signaling promotes Stk11s expression, and this proceeds through PKCθ- and hnRNPLL-mediated mechanisms. The data indicates Stk11s and Stk11l critically regulate Th17 versus iTreg cell fate choices, and that these, too, proceed downstream of functional PKCθ.

Altogether, we present compelling evidence that Stk11 alternative splicing and the LKB1 isoforms it produces, are central to mediating iTreg-Th17 cell plasticity. To this end, the present study may have implications for improving Treg cell therapies used to treat autoimmune diseases. A prevailing question in the field of Treg cell therapy is whether ex vivo-generated iTreg cells will resist adopting Th17-like effector phenotypes in a highly inflammatory environment (Bluestone, Tang, 2018). Our data show Stk11 splicing may lie at the heart of iTreg cell responses to IL-6 exposure and, thus, represent a unique target for developing stable iTreg cell-based therapies.
Figure 2.1. The short isoform of LKB1 is predominantly expressed in Th17 cells

CD4 T cells isolated were differentiated under Th17, iTreg, or non-polarizing (NP) conditions for 7 days prior to harvesting. We utilized flow cytometry to assess a) the percent of LKB1 positive cells within each cell subset and b) LKB1 protein expression, as measured by median fluorescence intensity (MFI). c) We visualized LKB1s expression by immunoblotting and quantified LKB1s expression relative to NP. Vinculin is shown as a loading control. We amplified the d) short and e) long splice variants of Stk11 using qRT-PCR, as well as f) the ratio of Stk11s to Stk11l transcript expression. Stk11 transcripts were quantified using the 2^-ΔΔCt method, normalized to ActB (β-actin), and are expressed relative to NP. Protein band intensity was quantified using ImageJ. Data are the mean of three independent experiments. Statistical analyses represented by symbols refer to comparisons between cells derived from WT and PKCθ^-/- mice, subjected to identical treatments. $ or *p < 0.05; & or **p < 0.01; ***p < 0.001; # or **** p < 0.0001; two-way ANOVA with Bonferroni correction applied.
Figure 2.2

Figure 2.2. HnRNPLL associates with Stk11S in Th17 cells and requires PKC0 for its expression
CD4 T cells isolated from WT mice were differentiated under Th17, iTreg, or non-polarizing (NP) conditions for 7 days prior to harvesting. a) We assessed hnRNPLL expression by immunoblotting and b) Hnrnpll levels by qRT-PCR. Vinculin is shown as a loading control. c) For RNA-IP, hnRNPLL was immunoprecipitated from whole cell lysate with RNase inhibitor to preserve RNA content. RNA was extracted from the lysate after immunoprecipitation, and we used qRT-PCR to amplify Stk11S transcripts. Hnrnpll transcripts were quantified using the $2^{-\Delta\Delta Ct}$ method, normalized to ActB (β-actin), and are expressed relative to the WT NP sample. Stk11S is expressed as bound units (BU) relative to Stk11S bound to hnRNPLL immunoprecipitated from NP samples. Protein band intensity was quantified using ImageJ. Data are the mean ± S.E.M of three independent experiments. Statistical analyses represented by symbols refer to comparisons between cells derived from WT and PKCθ⁻/⁻ mice, subjected to identical treatments. $ or *p < 0.05; & or **p < 0.01; ***p < 0.001; # or **** p < 0.0001; two-way ANOVA with Bonferroni correction applied.
Figure 2.3

PKCθ regulates Stk11 splice variant and LKB1 isoform expression upstream of hnRNPLL

CD4 T cells isolated from WT or PKCθ−/− mice were differentiated under Th17, iTreg, or non-polarizing (NP) conditions for 7 days prior to harvesting. We used qRT-PCR to quantify a) Prkcq and b) Hnrnpll transcript levels in WT Th17 and iTreg cells, as well as the levels of c) Stk11s and Stk11l. Relative gene expression was determined using the 2−ΔΔCT method. For Il17f and Prkcq, results are presented as fold expression of the gene of interest normalized to the housekeeping gene, ActB (β-actin), for each sample, and expressed relative to the Stk11 splice variants, the results are presented as percent of the variant expressed relative to total Stk11 (common) for each sample. d) We used immunoblotting to assess differences in LKB1 isoform in differentiated CD4 T cells from WT and PKCθ−/− mice. Vinculin is shown as a loading control. Protein band intensity was quantified using ImageJ. Data are the mean ± S.E.M of three independent experiments. Statistical analyses represented by symbols refer to comparisons between cells derived from WT and PKCθ−/− mice, subjected to identical treatments. $ or *p < 0.05; & or **p < 0.01; ***p < 0.001; # or **** p < 0.0001; two-way ANOVA with Bonferroni correction applied.
Figure 2.4

**Figure 2.4. HnRNPLL regulates Stk11s expression**
CD4 T cells isolated from WT or PKCθ<sup>−/−</sup> mice were differentiated under Th17, iTreg, or non-polarizing (NP) conditions for 7 days prior to harvesting. On day 3 of the differentiation process, Th17 polarized cells were transfected with 50 μM of Hnrnpll siRNA (siLL) or with a scrambled control (siScr). At the end of the differentiation period, we used qRT-PCR to quantify a) Stk11S and b) Stk11L. c) LKB1 isoform expression was assessed by immunoblotting. Vinculin is shown as a loading control. We also quantified d) Il17f and e) Prkcq by qRT-PCR. Relative gene expression was determined using the 2<sup>−ΔΔCT</sup> method. For Stk11 splice variants, the results are presented as percent of the variant expressed relative to the total Stk11 (common) for each sample. For Il17f and Prkcq, results are presented as fold expression of the gene of interest normalized to the housekeeping gene, ActB (β-actin), for each sample, and is expressed relative to the WT NP sample. Data are the mean ± S.E.M of three independent experiments. Statistical analyses represented by symbols refer to comparisons between cells derived from WT and PKCθ<sup>−/−</sup> mice, subjected to identical treatments. $ or *<i>p < 0.05</i>; & or **<i>p < 0.01</i>; ***<i>p < 0.001</i>; # or ****<i>p < 0.0001</i>; two-way ANOVA with Bonferroni correction applied.
Figure 2.5

CD4 T cells isolated from WT or PKCθ−/− mice were differentiated under Th17, iTreg, or non-polarizing (NP) conditions for 7 days prior to harvesting. iTregs were dosed with 20ng/mL of IL-6 on day 5 of the differentiation process, then harvested 48 hours later. We used qRT-PCR to quantify a) Prkcq, b) Hnrnpll, and d) Rorc and relative gene expression was determined using the 2−ΔΔCt method. The results are presented as fold expression of the gene of interest normalized to the housekeeping gene, ActB (β-actin), for each sample, and is expressed relative to the WT NP sample. c) Stk11s is expressed as a percent of the total Stk11 (common) for each sample. e) In vitro differentiated iTregs from WT or PKCθ−/− mice were labeled with Red650 and mixed with Ultra Green labeled responder cells (CD4 T cells) at three different ratios. Percent suppression was determined as described in Materials & Methods. Data are the mean ± S.E.M of three independent experiments. Statistical analyses represented by symbols refer to comparisons between cells derived from WT and PKCθ−/− mice, subjected to identical treatments. $ or *p < 0.05; & or **p < 0.01; ***p < 0.001; # or **** p < 0.0001; two-way ANOVA with Bonferroni correction applied.
Figure 2.6

**Figure 2.6. Stk11 splicing proceeds through a STAT3-mediated pathway**

CD4 T cells isolated from WT or PKCθ−/− mice were differentiated under Th17, iTreg, or non-polarizing (NP) conditions for 7 days prior to harvesting. iTregs were dosed with 20ng/mL of IL-6 on day 5 of the differentiation process. For some cultures, iTregs were also treated with 10 μM of the specific STAT3, Stattic, on days 5 and 6 of polarization. All cells were harvested on day 7. We used qRT-PCR to quantify a) Prkcq and b) Hnrnpll using the $2^{-\Delta\Delta Ct}$ method. The results are presented as fold expression of the gene of interest normalized to the housekeeping gene, ActB (β-actin), for each sample, and is expressed relative to levels in WT NP sample. We used flow cytometry to assess the percentage of c) IL17-expressing or e) FOXP3-expressing cells generated for each treatment condition and the median fluorescence intensity (MFI) of d) IL17 or f) FOXP3 expressed by each population. Data are the mean ± S.E.M of three independent experiments. Statistical analyses shown in grey refer to comparisons between conditions for cells derived from PKCθ−/− mice. Statistical analyses represented by symbols refer to comparisons between cells derived from WT and PKCθ−/− mice, subjected to identical treatments. $ or *p < 0.05; & or **p < 0.01; ***p < 0.001; # or **** p < 0.0001; two-way ANOVA with Bonferroni correction applied.
Figure 2.7. *Stk11* expression is sufficient to induce Th17 programming

CD4 T cells were isolated from WT or PKCθ−/− mice. Prior to differentiation, cells to be differentiated into Th17 cells were transduced with empty vector (Th17+EV) or with *Stk11* (Th17+*Stk11*)L, while cells to be differentiated into iTreg cells were transduced with empty vector (iTreg+EV) or with *Stk11* (iTreg+*Stk11*)S. At the end of the 7-day polarization process a) LKB1 expression was visualized by immunoblotting. Vinculin is shown as a loading control. We quantified b) Foxp3, c) Rorc, and d) Il17f by qRT-PCR. Relative gene expression was determined using the 2^−ΔΔCt method. The results are presented as the fold expression of the gene of interest normalized to the housekeeping gene, ActB (β-actin), for each sample and is expressed relative to the WT NP sample. Protein band intensity was quantified using ImageJ. Data are the mean ± S.E.M of three independent experiments. Statistical analyses shown in grey refer to comparisons between conditions for cells derived from PKCθ−/− mice. Statistical analyses represented by symbols refer to comparisons between cells derived from WT and PKCθ−/− mice, subjected to identical treatments. $ or *p < 0.05; & or **p < 0.01; ***p < 0.001; # or **** p < 0.0001; two-way ANOVA with Bonferroni correction applied.
Figure S1. LKB1 isoforms are generated from one of two Stk11 splice variants
CD4 T cells were differentiated under Th17, iTreg, or non-polarizing (NP) conditions and harvested after 7 days. a) Polarization efficiency was determined using flow cytometry by measuring the percent of cells that expressed RORγt or FOXP3. b) Cartoon depiction of LKB1/STK11 isoforms/splice variants. c) Diagram showing how the Stk11s splice variant is generated by incorporating the cryptic exon 9a, which has a stop codon at the end of the exon and changes the 3’UTR of the transcript. d) Cartoon depiction primer design strategy for amplifying Stk11s, Stk11L, and Stk11 common transcripts.
Figure S2

**Figure S2. HnRNPLL regulates Stk11s expression**

CD4 T cells from WT or PKCθ−/− mice were differentiated under Th17, iTreg, or non-polarizing (NP) conditions. On day 3 of the differentiation process, Th17 polarized cells were transfected with 50 μM of Hnrnpll siRNA (Th1+siLL) or Scrambled siRNA (Th17+siScr). On day 7, cells were harvested, and we quantified a) Hnrnpll by qRT-PCR. Relative gene expression was determined using the 2−ΔΔCT method. The results are presented as the fold expression of the gene of interest normalized to the housekeeping gene, ActB (β-actin), for each sample and is expressed relative to the WT NP sample. b) We visualized HnRNPLL using immunoblotting. Vinculin is shown as a loading control. Protein band intensity was quantified using ImageJ. c) In vitro differentiated iTregs from WT or PKCθ−/− mice, transduced with si-Hnrnpll or Scrambled siRNA as described above, were labeled with Red650 and mixed with Ultra Green labeled responder cells (CD4 T cells) at three different ratios. Percent suppression was determined as described in Materials & Methods. Data are the mean ± S.E.M of three independent experiments. Statistical analyses shown in grey refer to comparisons between conditions for cells derived from PKCθ−/− mice. Statistical analyses represented by symbols refer to comparisons between cells derived from WT and PKCθ−/− mice, subjected to identical treatments. **p < 0.01; ***p < 0.001; # or ****p < 0.0001; two-way ANOVA with Bonferroni correction applied.
**Figure S3. Stk11 splicing proceeds through a STAT3-mediated pathway**

CD4 T cells isolated from WT or PKCθ⁻/⁻ mice were differentiated under Th17, iTreg, or non-polarizing (NP) conditions for 7 days prior to harvesting. iTregs were dosed with 20ng/mL of IL-6 on day 5 of the differentiation process. For some cultures, iTregs were also treated with 10 μM of the specific STAT3, Stattic, on days 5 and 6 of polarization. All cells were harvested on day 7. We visualized a) hnRNPLL and e) LKB1 isoform expression by immunoblotting. Vinculin is shown as a loading control. Protein band intensity was quantified using ImageJ. b) We quantified IL17f by qRT-PCR. Relative gene expression was determined using the 2⁻ΔΔCt method. The results are presented as the fold expression normalized to the housekeeping gene, ActB (β-actin) for each sample and is expressed relative to levels in the WT NP sample. We used flow cytometry to assess c) the percentage of RORγt expressing cells generated for each treatment condition as well as d) the median fluorescence intensity (MFI). Statistical analyses shown in grey refer to comparisons between conditions for cells derived from PKCθ⁻/⁻ mice. Statistical analyses represented by symbols refer to comparisons between cells derived from WT and PKCθ⁻/⁻ mice, subjected to identical treatments. *p < 0.05; & or **p < 0.01; ***p < 0.001. # or ****p < 0.0001; two-way ANOVA with Bonferroni correction applied.
Figure S4

**Figure S4. Stk11s expression is sufficient to induce Th17 programming**

CD4 T cells were isolated from WT or PKC\(\theta^-\) mice. Prior to differentiation, cells to be differentiated into Th17 cells were transduced with \textit{Stk11} \(\text{L} \) (Th17+\textit{Stk11} \(\text{L} \)) or with empty vector (Th17+EV) while cells to be differentiated into iTreg cells were transduced with \textit{Stk11s} (iTreg+\textit{Stk11s}) or with empty vector (iTreg+EV). At the end of the 7-day polarization process we used qRT-PCR to quantify a) \textit{Stk11s} and b) \textit{Stk11L} using the 2\(^{-}\Delta\Delta Ct\) method. \textit{Stk11s} and \textit{Stk11L} are expressed as a percent of the total \textit{Stk11} (common) for each sample. Data are the mean ± S.E.M of three independent experiments. Statistical analyses shown in grey refer to comparisons between conditions for cells derived from PKC\(\theta^-\) mice. Statistical analyses represented by symbols refer to comparisons between cells derived from WT and PKC\(\theta^-\) mice, subjected to identical treatments. \(\& p < 0.05; *** p < 0.001; \# \text{ or } **** p < 0.0001; \) two-way ANOVA with Bonferroni correction applied.
CHAPTER 3

Interactions between SIRT1, PKCθ and LKB1 and their role in iTreg-Th17 plasticity

3.1 Introduction

Regulatory T cells are a subset of differentiated T helper cells that play a critical role in immunosuppression. In autoimmunity, Tregs are negatively regulated by the inflammatory cytokine milieu that inhibits their function. Th17 cells represent, in addition to Th1 and Th2, an independent helper T cell lineage. Th17 cells can be found under homeostatic conditions, particularly in the lamina propria of the small intestine (Kleinewietfeld et al., 2013). However, during infection or under inflammatory conditions, Th17 cells are induced in other tissues. CD4 T cells can differentiate into various effector cell populations with specialized function. This subset specific differentiation depends on numerous signals and the strength of stimulation. However, recent data have shown that differentiated CD4 T cell subpopulations display a high level of plasticity and that their initial differentiation is not an endpoint of T cell development.

The main striking difference between iTreg and Th17 cells are their distinct metabolic phenotypes. iTreg cells mainly use fatty acid oxidation (FAO), a catabolic process, to provide energy (Galgani et al., 2015). Th17 cells on the other hand utilize glycolysis, the pentose phosphate pathway, oxidative phosphorylation (OXPHOS), the hexosamine pathway, and fatty acid synthesis (FAS) as their means to provide energy. Thus, T cells can swiich between anabolism and catabolism by undergoing dramatic
metabolic changes. Identifying regulators of metabolic status may lay the foundation for developing therapeutics to modulate the immune response specifically in the context of autoimmune disorders.

In our study we are interested in factors that lead to the trans-differentiation from T regulatory (iTreg) cells to Th17 cells. This phenomenon is ubiquitous in autoimmune diseases such as Inflammatory Bowel Disease (IBD), which is characterized by an increase in inflammatory Th17 cells and a deficiency in iTreg cells. Sirtuin 1 (Sirt1) plays a crucial role in metabolism and inflammatory responses. Sirt1 is a deacetylase that can regulate different transcription factors important for modulating immune responses. The gut microenvironment produces high levels of the iTreg-inducing cytokine, TGFβ. However, when TGFβ is combined with pro-inflammatory IL6, which is often elevated with IBD, naïve CD4 T cells differentiate towards Th17 cells instead of iTregs (Galgani et al., 2015). Within this context, the deacetylase, Sirt1, has been shown to destabilize Foxp3, reducing the threshold for iTreg to Th17 conversion under pro-inflammatory conditions, such as in the presence of elevated IL6. Furthermore, blocking the actions of Sirt1 in iTregs increased their suppressive function both in vitro and in vivo (Galgani et al., 2015). These data suggest Sirt1 is an attractive target for enhancing iTreg cell differentiation and reducing the interconversion of iTregs towards a Th17 phenotype, which is especially important in IBD. LKB1, encoded by Stk11, has been identified as a mediator of Treg induction and function through its effects on TSDR methylation and metabolism. Sirt1 has been shown to activate LKB1, but the molecular mechanisms of these signaling pathways are not known.
We previously demonstrated that, a T cell-specific kinase, PKC\(\theta\), is sequestered in the cytosol away from the immunological synapse in Tregs, but not in Teff cells, implying that different localization of PKC\(\theta\) mediates different functions (Galgani et al., 2015). Inhibiting PKC\(\theta\) exerted differential effects on Tregs and Teffs (Galgani et al., 2015). Furthermore, inhibiting PKC\(\theta\) activity showed robust IS stabilization, leading to enhanced Treg cell suppressive function (Galgani et al., 2015). Although Tregs from PKC\(\theta\)-deficient mice were equally suppressive as Tregs from WT mice, they were not as stable as WT Tregs, suggesting that PKC\(\theta\) maintains the long-term stability of Treg cells. These findings support the idea that inhibiting PKC\(\theta\) in Tregs may be a substantial step towards Treg immunotherapy to treat autoimmunity.

Several groups have demonstrated that perturbing metabolic pathways through pharmacological manipulation can affect T cell phenotype providing a potential link between metabolism and plasticity (Gualdoni et al., 2016; Ozay et al., 2018; Wang et al., 2016). This effect has been extensively studied in Th17-iTreg plasticity. Our data suggest Stk11 splicing may lie at the heart of iTreg responses to IL-6 exposure and may represent a unique target for developing stable, cell-based iTreg therapies. We noted distinct differences in the isoform expression of the metabolic regulator LKB1 in Th17 and iTreg cells correlating with plasticity. Therefore, we predict that Sirt1 could interact with other proteins like, PKC\(\theta\) and LKB1, impairing or enhancing iTreg or Th17 differentiation. Examining the contribution of Sirt1 to this process may provide additional insight as to how LKB1 isoforms regulate iTreg-Th17 cell fate decisions.

3.2 Results

3.2.1 Sirt1-LKB1 interaction is more robust in Th17 cells compared to iTregs
LKB1, PKC0, and Sirt1 have been reported to only reside in the nucleus or the cytoplasm (Zhu et al., 2009). To confirm were these proteins localize in our T cell subsets after differentiation, we performed cytoplasmic vs nuclear extract and found that LKB1, PKC0, and Sirt1 are cytoplasmic proteins in NP, iTregs, and Th17 cells after day 7 of harvest (Figure 3.1A). Sirt1 was shown to play a role in Th17 differentiation and Sirt1 has been shown to activate LKB1, but the molecular mechanisms of these signaling pathways are not known (Kong et al., 2012). Our hypothesis is that the interaction between Sirt1 and LKB1 may be context specific and thus its interactions may affect T cell differentiation. To determine if LKB1 and Sirt1 interact to promote Th17 differentiation, we isolated CD4 T cells from wild-type mouse spleen and differentiated them into Th17 cells, and after 7 days harvested these cells. The controls were NP (Non-Polarized) and iTreg cells. To check for their interaction, we performed immunoprecipitation (IP) using either anti-Sirt1 or anti-LKB1, then immunoblotted with anti-SIRT1, anti-LKB1, anti-PKC0, and anti-Vinculin (as a loading control) to examine if these proteins interact. To further confirm these protein interactions, we performed AMNIS imaging flow cytometry. The protein expression was quantified using Fiji software. Each condition was normalized to the immunoprecipitated protein and expressed relative to NP condition. Our quantified immunoblot data suggested that Sirt1-LKB1 interactions are significantly greater in Th17 than in iTreg cells (Figure 3.1B). Interestingly, in all three conditions, the interactions between Sirt1-LKB1 were only observed with the LKB1 long isoform and not short isoform. Using the IDEAS software, we further confirmed that Sirt1 and LKB1 colocalization is greater in Th17 (Figure 3.1). Altogether, our data suggests a differential interaction between Sirt1-LKB1 in Th17 and iTreg cells.
3.2.2 PKCθ-Sirt1 interaction is more robust in iTregs cells compared to 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). Sirt1 is a deacetylase, and Li et al., observed in their study that Sirt1 regulates the acetylation of PKCθ, thereby altering its phosphorylation and activation. Therefore, we asked whether PKCθ and Sirt1 might differentially associate Th17 vs iTregs. To check for their interaction, we performed immunoprecipitation (IP) both of Sirt1 and PKCθ, and immunoblotted with anti-Sirt1, anti-LKB1, anti-PKCθ, and anti-Vinculin (as a loading control) to determine if these proteins interact. To further confirm protein interactions, we performed AMNIS imaging flow cytometry. The protein expression was quantified using Fiji software. Each condition was normalized to the immunoprecipitated protein and expressed relative to NP condition. Our quantified immunoblot data suggested that PKCθ-Sirt1 interaction is more robust in iTregs cells compared to Th17 (Figure 3.2A). Using the IDEAS software, we further confirmed that PKCθ and Sirt1 colocalization is greater in iTregs (Figure 3.2B). Altogether, our data suggests a differential interaction between PKCθ-Sirt1 in Th17 and iTregs. Our next question was to investigate if PKCθ aids in establishing the interaction between Sirt1 and LKB1. To answer this question, we isolated naive CD4 T cells from PKCθ−/− mice, stimulated, and cultured them under NP, iTreg or Th17 polarizing conditions. On day 7 of harvest, we performed immunoprecipitation (IP) with anti-Sirt1, and immunoblotted with anti-SIRT1, anti-LKB1, anti-PKCθ, and anti-Vinculin (as a loading control) to examine if these proteins interact. We observed from the quantified immunoblot that, Sirt1-LKB1 interaction...
interactions are reduced in Th17 cells in the absence of PKCθ in comparison to iTregs, confirming PKCθ is necessary for Sirt1-LKB1 interaction in Th17 cells (Figure 3.2C).

3.2.3 PKCθ-LKB1 interactions are more robust in Th17 cells compared to iTregs

LKB1 is a serine-threonine protein kinase, when inhibited, results in unregulated cell growth and tumor formation. However, how LKB1 is regulated remains poorly understood (Ping et al., 2007). LKB1 signaling is regulated through two main mechanisms: phosphorylation and subcellular localization (Ping et al., 2007). LKB1 activity is regulated by the formation of complexes with STRAD and MO25 (Boudeau et al., 2003). In the absence of these proteins, overexpressed LKB1 is localized to the nucleus. Formation of the LKB1·MO25·STRAD complex causes LKB1 to localize the cytosol and enhances LKB1 activity (Boudeau et al., 2003). In endothelial cells, LKB1 activates AMP-activated protein kinase in response to reactive nitrogen species or metformin through a protein kinase C (PKC)-dependent mechanism (Xie et al., 2006). Thus, we wanted to investigate whether PKCθ acts upstream of LKB1 to regulate Th17 or iTreg differentiation. We performed immunoprecipitation (IP) of both Sirt1 and PKCθ, and immunoblotted with anti-SIRT1, anti-LKB1, anti-PKCθ, and anti-Vinculin (as a loading control) to examine if these proteins interact. To further confirm these protein interactions, we performed AMNIS imaging flow cytometry. The protein expression was quantified using Fiji software. Each condition was normalized to the immunoprecipitated protein and expressed relative to NP condition. Our quantified immunoblot data suggested that PKCθ and LKB1 interact significantly more in Th17 than in iTreg cells (Figure 3.3A). Interestingly, in all three conditions, the interactions between Sirt1 and LKB1 were only observed with the LKB1
long isoform and not short isoform. Using the IDEAS software, we further confirmed that PKCθ and LKB1 colocalization is greater in Th17 cells (Figure 3.3B). Altogether, our data suggests a differential interaction between PKCθ-LKB1 in Th17 and iTregs.

Our next question was to investigate if LKB1 and its isoforms (long and short) are necessary to aid the interaction between Sirt1-PKCθ in Th17 and iTreg differentiation. LKB1 has been shown to be important for thymocyte development (Cao et al., 2010). Thus, we needed to incorporate a Tamoxifen (TAM)-inducible Cre/loxP to delete Stk11 from Stk11 floxed mice. For our preliminary studies, we wanted to test if we can delete LKB1 in vitro. To do this, we purchased a Cre-GFP plasmid (addgene) and cloned it into the retroviral vector PMX-GFP-IRES. We then retrovirally transduced CD4 T cells that were stimulated and plated in 12-well plates. Transduction media was replaced with Th17- or iTreg-polarizing culture media 4 h after spin-infection, and the cells were harvested on Day 3. We used pMX-EV as our empty vector control. We observed that retroviral transduction with Cre deletes Stk11 expression in our murine cells (Figure 3.3C).

3.3 Discussion

In this study, we asked whether there was an interaction between Sirt1, LKB1 and PKCθ, in relation to their differentiation into Th17 or iTreg cells. We showed that Sirt1 and LKB1 interact differently in Th17 and Tregs. This interaction might be important for establishing the Th17 phenotype. Furthermore, PKCθ aids in establishing the interaction between Sirt1-LKB1 in Th17 cells. We also showed that Sirt1, LKB1 and PKCθ are all cytoplasmic proteins. We demonstrated that in our differentiated cell-types, NP, iTreg and Th17, retroviral transduction with Cre deletes Stk11, in vitro, in Stk11 floxed mice.
Previous studies demonstrated that activation of AMPK/Sirt1 signaling through LKB1 regulates the balance between Th17 and Treg cells and that therapeutic strategies targeting the Th17/Treg balance via the AMPK/Sirt1 pathway might be beneficial for the treatment of autoimmune diseases like Necrotizing enterocolitis (NEC; Ma et al., 2020). Our data shows Sirt1 and LKB1 can physically interact in Th17 cells and that this interaction is PKCθ-dependent. Further experiments are needed to determine whether this physical interaction is independent of AMPK signaling.

Sirt1, a histone/protein deacetylase, and LKB1 are key enzymes involved in longevity and energy homeostasis (Lan et al., 2008). Sirt1 deacetylates LKB1 and this is associated with its movement to the cytoplasm where it is bound to and activated by STRAD (Lan et al., 2008). 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), while also acting in pathways to inhibit glycolysis (Ma et al., 2017). 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). Thus, our results beg the question: Is Sirt1 and LKB1 physical interaction necessary for establishing the Th17 phenotype and how does PKCθ facilitate this interaction? Our
data shows that Sirt1 interacts with the LKB1 long isoform. How this affects Th17 vs iTreg cell differentiation remains to be elucidated. What we can conclude, however, is that Sirt1 differentially interacts with other proteins, PKCθ and LKB, in iTreg and Th17 cells.
Figure 3.1: Sirt1-LKB1 interaction is more robust in Th17 cells compared to iTreg cells.

CD4 T cells isolated from wild type mice were differentiated under Th17, iTreg, or non-polarizing (NP) conditions for 7 days prior to harvesting. (A) Cytoplasmic and nuclear extracts were isolated from WT CD4 T cells were differentiated into NP or Th17 cells and probed for anti-Sirt1, anti-LKB1, anti-PKC0, anti-Vinculin (as a loading control) and anti-HDAC for nuclear protein loading control. (B) We utilized co-immunoprecipitation with Sirt1 or LKB1 to quantify, using the Fiji software, Sirt1-LKB1 and LKB1-Sirt1 interaction by immunoblotting with anti-Sirt1, anti-LKB1, anti-PKC0, and anti-Vinculin (as a loading control). (C) We utilized AMNIS imaging flow cytometry to detect differences in LKB1 and Sirt1 colocalization. AMNIS images of NP, iTregs and Th17 cells. Sirt1 is in green, LKB1 is in blue, and Draq5 is in red (nuclear marker). The percent colocalized was determined using IDEAS software. Data are the mean of three independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001; unpaired, two-tailed Student’s t-test.
Figure 3.2: PKCθ-Sirt1 interaction is more robust in iTreg cells compared to Th17 cells. CD4 T cells isolated from wild type mice were differentiated under Th17, iTreg, or non-polarizing (NP) conditions for 7 days prior to harvesting. (A) We utilized co-immunoprecipitation with anti-Sirt1 or anti-PKCθ to quantify, using Fiji software, PKCθ–Sirt1 and Sirt1-PKCθ interactions by immunoblotting with anti-SIRT1, anti-LKB1, anti-PKCθ, and anti-Vinculin (as a loading control). (B) We utilized AMNIS imaging cytometry to detect differences in PKCθ and Sirt1 colocalization. AMNIS images of NP, iTregs and Th17 cells. Sirt1 is in green, PKCθ is in blue, and Draq5 is in red (nuclear marker). The percent colocalized was determined using the IDEAS software. (C) Naive CD4 T cells from PKCθ−/− mice were isolated, stimulated and cultured under NP, iTreg and Th17 polarizing conditions. On day 7 of harvest, we performed immunoprecipitation (IP) with anti-Sirt1, and immunoblotted with anti-Sirt1, anti-LKB1, anti-PKCθ, and anti-Vinculin (as a loading control) and quantified the interactions using the Fiji software. Data are the mean of three independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001; unpaired, two-tailed Student’s t-test.
Figure 3.3

PKCθ-LKB1 interactions are more robust in Th17 cells compared to iTregs. CD4 T cells isolated from were differentiated under Th17, iTreg, or non-polarizing (NP) conditions for 7 days prior to harvesting. (A) We utilized co-immunoprecipitation with anti-LKB1 or anti-PKCθ to quantify, using the Fiji software, PKCθ–LKB1 and LKB1-PKCθ interactions by immunoblotting with anti-SIRT1, anti-LKB1, anti-PKCθ, and anti-Vinculin (as a loading control). (B) We utilized AMNIS imaging cytometry to detect differences in PKCθ and LKB1 colocalization. AMNIS images of NP, iTregs and Th17 cells. PKCθ is in green, LKB1 is in blue, and Draq5 is in red (nuclear marker). The percent colocalized was determined the IDEAS software. (C) CD4 T cells were isolated from Stk11 floxed and WT mice. Stk11 floxed CD4 T cells were transduced with empty vector (LKB1+EV) or with cre-GFP (LKB1+cre) and harvested on day 3. WT CD4 T cells were differentiated under Th17, iTreg, or non-polarizing (NP) conditions for 7 days. LKB1 expression was visualized by immunoblotting. Vinculin was used as a loading control. Data are the mean of three independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001; unpaired, two-tailed Student’s t-test.
CHAPTER 4

4.1 Conclusions and future directions

We describe for the first time a connection between plasticity, and LKB1 isoform expression. LKB1 has been identified as a mediator of Treg induction and function through its effects on TSDR methylation and metabolism. We demonstrate that LKB1 is expressed in both Th17 and iTreg cells and to a much higher extent in Th17 cells. LKB1 has two isoforms: long and short; we have determined that LKB1s is predominately expressed in Th17 cells and LKB1l in iTreg cells. We provide evidence that the isoform expression is a mode of plasticity between the two phenotypes. As such, when IL-6, which is inhibitory to Tregs, is administered to iTregs, the short isoform is induced. Moreover, we demonstrate that IL6 also upregulates PKCθ in iTreg cells and the expression of PKCθ correlates with LKB1s expression. We give further evidence that PKCθ, in turn, regulates the expression of hnRNPLL and subsequent Stk11 splicing. Our data also showed that Stk11s and Stk11l critically regulate Th17 versus iTreg cell fate choices, and that these, too, proceed downstream of functional PKCθ. We also showed that Sirt1-LKB1 interact differently in Th17 and Tregs. This interaction might be important for establishing the Th17 phenotype. PKCθ, Sirt1 and LKB1 interact with each other in a tripartite complex and this interaction is differentially established in iTregs and Th17 cells. Thus, this work identifies for the first time a connection between IL6, PKCθ, and LKB1 associated with iTreg-Th17 cell plasticity, as well as differential interaction between PKCθ, LKB1, and Sirt1 in iTregs and Th17 cells.
How and whether metabolism may regulate T cell plasticity is important to consider. *In vivo* plasticity between Treg and Th17 cells has been demonstrated in autoimmune conditions such as colitis (Ren & Li, 2017), raising the possibility that disease pathology may be perpetuated as Tregs are converted into IL17-producing cells. Identifying key molecules that regulate the metabolic dynamics of Tregs and Th17 cells will aid in developing therapeutics to specifically modulate the ratio of effector to Treg cells. To this end, the present study may also have implications for improving Treg therapies to treat autoimmune diseases. A prevailing question in the field of Treg therapy is whether *ex vivo*-generated iTregs will resist adopting Th17-like effector phenotypes in a highly inflammatory environment (Ren et al., 2017). Our data suggest *Stk11* splicing may lie at the heart of iTreg responses to IL6 exposure and may represent a unique target for developing stable, cell-based iTreg therapies. Our proposed experiments examining the contribution of Sirt1 to this process may provide additional insight as to how, more specifically, LKB1 isoforms regulate iTreg-Th17 cell fate decisions.

4.2 Future Directions

Even though the discoveries described in this dissertation significantly advance our knowledge of IL6, PKCθ, LKB1 and Sirt1 mechanisms regulating T cell differentiation, they also raise many other questions that remain to be investigated, such as: (1) uncovering the molecular mechanisms governing hnRNPL regulation of *Stk11* splicing in iTregs, (2) determining if LKB1 offers Tregs phenotypic stability, (3) Determine if Sirt1 is required, and functions downstream of LKB1, to mediate Th17 cell differentiation, (4) Determine whether association of Sirt1 with LKB1s or LKB1L affects the suppressive capacity of iTreg cells.
4.2.1 Determine the molecular mechanisms governing hnRNPL regulation of Stk11 splicing in iTregs

In this study, we predominately focused on deducing the molecular mechanisms involved in the regulation of RBP, hnRNPLL in Stk11 splicing in Th17 cells. Heterogenous nuclear ribonuclear protein L (hnRPNL) 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. Thus, we hypothesized that hnRNPL may block the splicing of Stk11 short by blocking exon9a which is a cryptic exon, and preferentially bind to Stk11 long driving the iTreg phenotype. We demonstrated in our study that there are differences in Stk11 isoforms between Th17 and iTreg cells. We also observed differences in hnRNPL binding to Stk11 transcript in Th17 versus iTreg cells. Specifically, we observed higher expression of hnRNPLL in Th17 cells and higher expression of hnRNPL in iTregs. Our data also highlighted the necessity of hnRNPLL in Stk11 splicing. However, it remains unclear whether hnRNPL binding blocks or drives the splicing of Stk11, specifically Stk11L. To ask this question, we will need to conduct an antibody delivery experiment to hnRNPL or use an siRNA to hnRNPL and assess what happens to Stk11 transcript when hnRNPL is functionally inhibited. If there are lower levels of Stk11L, we can postulate that hnRNPL inhibits Stk11 splicing.

We next asked if, IL6 signaling can skew iTreg-Th17 cell balance. We observed that IL6 exposure downregulates Hnrnpl and upregulates Hnrnpll in WT iTregs
suggesting preferential expression of hnRNPL in iTregs. In our study, we demonstrated that PKC\(\theta\) regulates Stk11 expression through hnRNPLL. Additional studies are needed to further tease out how exactly PKC\(\theta\) regulates hnRNPL cellular localization. In Ozay et al. (2020) our group determined that nuclear hnRNPL is diminished in human iTreg cells treated with anti-phospho-PKC\(\theta\) (T538), delivered intracellularly, while the cytosolic localization of hnRNPL is increased. This suggests that PKC\(\theta\) 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 PKC\(\eta\) seems to be a regulator to Th17-iTreg plasticity, we hypothesized that hnRNPL localization would be primarily cytosolic in the absence of PKC\(\theta\) in iTreg cells. If hnRNPL is indeed inhibitory to Stk11\(\alpha\) expression, this finding could further explain why there is less Stk11\(\alpha\) expression in the absence of PKC\(\theta\). However, these data are preliminary and need to be further validated.

If hnRNPL does have a role in regulating Stk11\(\alpha\) expression, 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 PKC\(\theta\)-mediated phenomenon.

4.2.2 Does LKB1 provide phenotypic stability to Tregs?
Treg cells can be directly administered to treat autoimmune disease by way of polyclonal Tregs or Tregs transduced with a receptor with high affinity for the target autoantigen, such as a high affinity T cell receptor (TCR) or a chimeric antigen receptor (CAR) (Juneja et al., 2022). But a crucial obstacle in Treg therapy is destabilization of the Treg phenotype after transplantation. The concept of Treg cell stability, which is defined as the ability to maintain Foxp3 expression and resist acquiring pro-inflammatory effector functions during inflammation, has emerged as a crucial determinant of Treg cell function in selective contexts (Juneja et al., 2022). A key feature of Treg phenotypic stability is the methylation of the Treg-Specific Demethylation Region (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 Treg therapy is not entirely efficacious and may even exacerbate inflammation. This was reported in a study by Dall’Era and colleagues, where after 12 weeks of Treg therapy, there was an increase in IL17-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+IL17+ cells also increased, suggesting that the transplanted Tregs may be subject to conversion to IL17 producing cells, in vivo (Dall’Era et al., 2019). These data support the idea that these “stable” Tregs cells do not necessarily remain phenotypically stable over time, especially following transplantation.

LKB1 has been shown to protect Treg function by maintaining metabolism, cellular survival, and Foxp3 expression (Wu et al., 2017). Wu et al., reported that LKB1 blocks STAT 4-mediated methylation of the TSDR, to promote stable Tregs. Additionally, LKB1+Tregs failed to suppress aberrant immune responses resulting in systemic autoimmunity.
(Wu et al. 2017). Foxp3 expression is dependent on LKB1 expression, as delivering an shRNA against stk11 reduced Foxp3 expression, while overexpressing stk11 increased Foxp3 expression (Su et al., 2019). LKB1 was shown to regulate the differentiation and activation of T effector cells. In LKB1-deficient mice, T effector cells display altered activation and differentiation, leading to reduced immune function and increased susceptibility to infections and tumors, and higher production levels of cytokines. This could possibly be a result from faulty thymocyte development, enhanced effector T cell function, or diminished Treg suppression (MacIver et al., 2011). However, to our knowledge how LKB1 may contribute to Treg-Th17 plasticity has not been explored.

Regulatory T cells (Tregs) play a crucial role in preventing GVHD by suppressing the immune response of effector T cells and maintaining immune tolerance. Tregs exert their immunosuppressive function through various mechanisms, including the secretion of cytokines and the expression of inhibitory molecules. Recent studies have suggested that LKB1 may play a role in the regulation of Treg function and prevention of GVHD. In LKB1-deficient mice, Treg function is reduced, leading to increased immune response and increased susceptibility to GVHD. These patients’ Tregs showed reduced functionality which correlated with low LKB1 expression in Tregs (Su et al., 2019). Thus, LKB1 plays a crucial role in the regulation of Treg function, making it an attractive target for therapeutic approach in treating autoimmune disorders.

In this study our data implicates that splice variants of stk11 act as mediators 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. In our study, we demonstrate that downstream of IL6, a
cytokine that induces IL17 secretion and inhibits Foxp3 expression, PKCθ and hnRNPLL act in association, to induce LKB1S expression. We also observe higher expression of LKB1L in our iTregs. This leads to the question of whether blocking LKB1 splicing into its short isoform, or overexpression of the long isoform results in more stable Tregs.

To test the effect of LKB1L on Treg stability we can first knockout LKB1L in Tregs and assess cytokine production. To do this, we can design an siRNA to the long form, perform a transfection in our differentiated cells, and assess expression of the Foxp3 transcription factor. Specifically, is there a correlation between LKB1L expression and the TSDR methylation? This can be evaluated using bisulfite sequencing. If we correlate LKB1L isoform expression with Treg stability, can we target this pathway to stabilize the Treg phenotype? To do this, we would overexpress the long isoform using retroviral transduction and assess the expression of Foxp3.

A second approach to potentially produce more stable Tregs *ex vivo* could be achieved by blocking LKB1 splicing into its short form. To test the effect of LKB1s on Treg stability we can first knockout LKB1s in Tregs and assess cytokine production. Preliminary work done in our lab, showed that complexing polymers with antibodies such as anti-pPKCθ or anti-Sirt1 into CD4 T cells prior to differentiating them into iTregs makes them more potent suppressors, both *in vitro* and *in vivo*. In this study, we have demonstrated that PKCθ is induced by the proinflammatory cytokine, IL6, and acts upstream to regulate LKB1 splicing. Thus, we hypothesized that inhibiting PKCθ using an antibody delivery strategy may make iTreg cells more resistant to destabilization when provided with IL6. To answer this question, we would deliver anti-PKCθ to iTregs prior to stimulation and add IL6 on day 5. To assess for iTreg stability, we would compare the TSDR
demethylation patterns, \textit{Stk11} splice variants, and proinflammatory cytokine production in anti-pPKC\(\theta\)-treated and untreated iTregs in the presence of IL6. If anti-pPKC\(\theta\) treatment increased iTreg stability, we would expect to see protected TSDR demethylation, reduced \textit{Stk11}s (or relative increases in \textit{Stk11L}), and reduced IL17 secretion.

\textbf{4.2.3 Determine if Sirt1 is required, and functions downstream of LKB1, to mediate Th17 cell differentiation.}

Studies have shown Sirt1 and LKB1 interact with each other (Chen et al., 2022, Wang et al., 2022). In mammals, Sirt1 acts as an epigenetic regulator that modulates the activity of several transcription factors important for immune function (Kwon et al., 2012, Zhang et al., 2008). Initial studies using mice with globally deleted \textit{Sirt1}, identified Sirt1 as having primarily an anti-inflammatory function (Zhang et al., 2008, Gao et al., 2012). More recent work focusing on T cells has identified Sirt1 as having proinflammatory actions, negatively regulating Treg cell function by deacetylating \textit{Foxp3}, the signature transcription factor of Treg cells (Chen et al., 2022, Wang et al., 2022, van Loosdregt et al., 2010). Conversely, Sirt1 has been shown to positively regulate Th17 cell function by modulating ROR\(\gamma\)t activity. \textit{In vivo}, \textit{Sirt1} deficiency resulted in impaired production of proinflammatory Th17 cells and reduced susceptibility to Th17 cell–mediated autoimmune disease. Here we will ask if there is a combinatorial role for LKB1 and Sirt1 to mediate Th17 cell differentiation.

To do this, we will use CD4 T cells from \textit{Stk11}\textsuperscript{\textminus\textminus} mice and delete LKB1 using an \textit{in vitro} TAT-cre system. Following LKB1 deletion, we will isolate CD4 T cells and differentiate them for 7 days into Th17 cells. The controls will be NP (Non-Polarized) and
iTregs, with LKB1 deleted, as well as wild-type Th17 cells. We will isolate protein from whole cell lysates and use immunoblotting to determine LKB1 (to confirm deletion) and Sirt1 expression. We will also inhibit Sirt1 in LKB1-deficient cells, using a specific Sirt1 inhibitor, and examine the different Th17 differentiation markers, Rorγt and IL17, using flow cytometry and rorc and Il17a, using RT-qPCR. We predict that if LKB1 is required for Sirt1-mediated Th17 differentiation, we expect that deleting LKB1 will diminish Th17 differentiation. If both LKB1 and Sirt1 are required for Th17 cell differentiation, this will be revealed in cells in which LKB1 is deleted and Sirt1 is inhibited.

4.2.4 Determine whether association of Sirt1 with LKB1s or LKB1L affects the suppressive capacity of iTreg cells.

Blocking Sirt1 was shown to generate Tregs with increased expression of Foxp3, potentially making them super suppressive as well (Velagapudi et al., 2017, Park et al., 2022). Previous studies have shown the importance of LKB1 in Treg stability (Sun et al., 2022, Grinberg-Bleyer et al., 2018), but that study did not consider the different isoform expression between Th17 and iTreg cells, that we have observed. iTregs cells show significantly greater expression both of Stk11L and LKB1L, while Th17 cells upregulate Stk11S and LKB1S. Thus, Sirt1’s association with a specific LKB1 isoform may affect the stability and suppressive capacity of Tregs. To test this hypothesis, we will use CD4 T cells from Stk11fl/fl mice and delete LKB1 using an in vitro TAT-cre system. Following LKB1 deletion, we will transduce cells either with Stk11S (LKB1S) or Stk11L (LKB1L) and differentiate cells towards an iTreg phenotype. The controls will be NP (Non-Polarized) and Th17 cells, in which LKB1 has been floxed, and LKB1s or LKB1L has been re-
expressed. We will plan to perform a suppression assay by co-culturing the iTreg cells, in various ratios, with stimulated CD4 T cells. On day 3 of the suppression assay we will plan to examine the suppressive capacity/expression of Treg markers using flow cytometry for each condition. We predict that if iTreg suppressive capacity requires Sirt1 to associate with LKB1L, we expect to see reduced suppression in LKB1-floxed iTregs transduced with Stk11s. Conversely, we would expect to see enhanced suppression in LKB1-floxed iTregs transduced with Stk11L.

Altogether, this dissertation establishes a central role for LKB1 isoforms in the regulation of T cell plasticity and highlights its interaction with other proteins such as PKCθ and SIRT1 in a tripartite complex. The studies outlined in this dissertation identified key molecules that regulate iTreg - Th17 plasticity in hopes for developing therapeutics to prevent iTregs from adopting a pro-inflammatory state in the future.
CHAPTER 5
Materials and Methods

5.1 Mice
All animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Massachusetts Amherst. Wild-type C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME), Stk11 floxed (stk11-fl/fl) mice, which carry an Stk11 allele with LoxP sites flanking exons 3 through 6 were obtained from The Jackson Laboratory.

5.2 In vitro T cell polarization
CD4 T cells were isolated from spleens with Mojosort™ (Biolegend, San Diego, CA). Cells were stimulated on plates coated with anti-hamster IgG (Sigma-Aldrich, St. Louis, MO) plus 1mg/ml anti-CD3 (clone 145-2C11; BioLegend, San Diego, CA) and 1mg/ml anti-CD28 (clone 37.51; BD Biosciences, San Jose, CA). iTreg cultures were stimulated with the same clones using plate bound anti-CD3 and soluble anti-CD28. Cells were incubated in a 1:1 mixture of RPMI and DMEM (GE Life Sciences, Pittsburgh, PA), supplemented with L-glutamine, sodium pyruvate, Penicillin-streptomycin (GE Life Sciences) and Fetal Bovine Serum (Peak Serum, Wellington, CO). IL-6 (20ng/ml), TGFβ (20ng/ml), and IL-2 (135U/ml), all from BioLegend were added to Th17-polarized cultures. 10mg/ml of anti-IFNγ (clone XMG 1.2; BioXcell) and 10mg/ml of anti-IL-4 (clone 41B11; BioLegend and BioXcell, West Lebanon, NH) and were added to the Th17 cultures to prevent Th1 or Th2 skewing, respectively. 2.5nM Retinoic acid (Sigma-Aldrich) was added to iTreg cultures. Cells were polarized in culture for 7 days after...
stimulation. For some experiments, CD4 T cells were polarized under iTreg or Th17 polarizing conditions and on days 5 and 6 of the differentiation process were treated with 10 μM of the specific STAT3 inhibitor, Stattic (Santa Cruz Biotechnology, Dallas, TX), according to the manufacturer’s recommendations. DMSO (1% in PBS) was added to untreated wells as a vehicle control. CD4 T cells were differentiated under non-polarizing (NP), iTreg, or Th17 polarizing conditions. On day 3 of the differentiation process, Th17 polarized cells were transfected with 50 μM of HnrnpLL siRNA, 2.5ul of X-tremeGene siRNA transfection reagent and 50ul of serum free media (SFM). After incubating the mixture for 20 minutes, it was added to cells plated in 12 well plates in a dropwise manner.

5.3 Flow cytometry

Antibodies used for staining are as follows: CD4 FITC (clone H129.19), CD25 APC (clone PC96), FOXP3 PE (FJK-16s), FOXP3 PE (clone 150D, all from BD Biosciences, San Jose, CA); LKB1 clone (D60C5), RORγt PE (clone AFKJS-9; from eBioscience, San Diego, CA), and F(ab’)2 IgG QDot625 (Life Technologies, Carlsbad, CA). Cells were stained with antibodies to surface markers using CD4 PE-Cy7 (GK1.5) and CD25 PE (clone M-A251, both from BioLegend) for 30 min on ice. For intracellular cytokine staining, cell suspensions were restimulated in vitro for a total of 4 h with 50 ng/mL phorbol 12-myristate 13-acetate (PMA) and 1 μM Ionomycin (both from Sigma-Aldrich) with addition of 2 μM monensin (eBioscience) to inhibit secretion. After surface marker staining, cells were stained with the Live/Dead Fixable Aqua Dead Cell Stain Kit (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions. Before intracellular staining, cells were fixed and permeabilized using the FOXP3/Transcription Factor Staining Buffer Set (eBioscience) according to the manufacturer's instructions. Antibodies
used for intracellular staining were FOXP3 Alexa Flour 700 (clone MF-14, BioLegend), RORγt (clone Q31-378, BD Biosciences), IL-17A (clone TC11-18H10, BioLegend). Samples were acquired using a BD LSR Fortessa flow cytometer (BD Biosciences) and data was analyzed using FlowJo software (BD Biosciences). Cells were processed using a Luminex ImageStreamx mkII imaging flow cytometer. Cells were fixed and permeabilized with the Foxp3/Transcription Factor Staining Buffer Set (eBioscience). Nuclei were stained using DRAQ5 (ThermoFisher Scientific) Analyses were made using IDEAS, (Luminex Corporation).

5.4 qRT-PCR
RNA was isolated from cells using the Quick-RNA Mini-Prep kit (Zymo Research, Irvine, CA). cDNA was synthesized with Oligo(dt) 12-18 primer (ThermoFisher Scientific), m-MLV reverse transcriptase and RNasin® plus inhibitor (Promega Corporation, Madison, WI), and dNTPs (New England Biolabs, Ipswich, MA). cDNA was used in qRT-PCR reactions with SYBR Green Master Mix (Bimake, Houston, TX). Reactions were conducted using an Eppendorf nexus X2 (Eppendorf, Framingham, MA). Data were analyzed using the $2^{-\Delta\Delta CT}$ method. Primer sequences (Integrated DNA Technologies, Coralville, IA) are listed in Supplemental Table 1.

5.5 Western blot
Cells were lysed in RIPA buffer in the presence of phosphatase and protease inhibitors (Bimake, Houston, TX). Antibodies used for immunoblotting: hnRNPLL, (Cell Signaling Technologies, Danvers, MA), Vinculin (clone VIN-11-5, Millipore Sigma, Burlington, MA), LKB1 (clone Ley37D/G6, Santa Cruz Biotechnologies). Secondary antibodies used:
anti-mouse IgG HRP (GE Amersham, Pittsburgh, PA) and anti-rabbit IgG HRP (Cell Signaling Technologies and GE Amersham).

5.6 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, Madison, WI). Dynabeads Protein G® were coated with hnRNPLL (Cell Signaling Technologies) at room temperature in a solution of 1% BSA (Rocky Mountain Biologicals, Missoula, MT) in PBS. Lysates and beads were incubated together and washed with IP buffer after incubation.

5.7 Plasmid extraction and purification

DH5α competent cells were transformed with Stk11 plasmids and amplified overnight in LB medium containing specific antibiotics using a 37°C shaker at 225 rpm. Bacteria were harvested and Stk11S and Stk11L plasmids were extracted and purified using a plasmid extraction and purification kit (Takara, San Jose, CA) according to the manufacturer’s protocol.

5.8 Cre-GFP, Stk11S and Stk11L Overexpression and Retroviral Transduction

Isolated Cre-GFP, Stk11S and Stk11L plasmids were then cloned into the pMRX-IRES-GFP vector, which contains a green fluorescent protein (GFP) reporter. Empty pMRX-IRES-GFP vectors were used as controls. Retroviral supernatants were produced by transfecting Platinum-E (Plate-E) retroviral packaging cells using Transporter 5 transfection reagent (Polysciences, Warrington, PA). Retroviral supernatants were concentrated 10x in lymphocyte culture media with PEG-it™ virus concentration reagent (System Biosciences,
Palo Alto, CA) prior to cell transduction. CD4 T cells were retrovirally transduced 24 h after activation with 10x-concentrated retrovirus supernatants by spin-infection (660 × g for 90 min at 37°C) in the presence of polybrene (4 μg/mL). Transduction media was replaced with Th17- or iTreg-polarizing culture media 4 h after spin-infection. Transduced cells were analyzed by immunoblotting or qRT-PCR.

5.9 In Vitro Suppression Assay

On day 0, murine CD4 T cells were plated onto anti-CD3 plus anti-CD28-coated wells and differentiated for 7 days in iTreg differentiation media. On day 7, iTregs (suppressors) were loaded with the cell tracker dye Red650 (allophycocyanin [APC] fluorescence). Total CD4 T cells (responders) were stimulated with soluble anti-CD3 plus anti-CD28 and crosslinked using mouse IgG. Responder cells were then loaded with a different cell tracker dye, UltraGreen (fluorescein isothiocyanate [FITC] fluorescence). Responder cells were seeded onto tissue culture plates and suppressors were added to responders at the indicated ratio. Cells were co-cultured for 3 days and proliferation of responder and suppressor cells was determined using flow cytometry. Percent suppression was calculated as:

\[
\text{area under the curve of Tresp (without Tregs) - area under the curve of Tresp (with Tregs)} \times 100
\]

area under the curve of Tresp (without Tregs)

5.10 Cytoplasmic and Nuclear Extract Isolation

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

Data shown are the mean ± SEM and all experiments were repeated at least three times. Unpaired, two-tailed Student’s t-test or two-way ANOVA with Bonferroni correction was applied for statistical comparisons using GraphPad Prism 8 software. \( P \) values of \( \leq 0.05 \) were considered significant.
### Supplemental Table 1

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**For Transduction:**

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| **Stk11L** | CATCGGGAATTGACGTGGCAGCCCGACAGGACAGGCACTG | GCCTGCCTCGAGCTACACTAAGCCCAAA |
| **Stk11S** | CATCGGGAATTGACGTGGCAGCCCGACAGGACAGGCACTG | CCCCTC |
Table 5.1 Antibodies used in this study

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