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The role of Cryptochrome in Regulatory T cell development and function

A dissertation submitted in partial satisfaction of the requirements for the degree

Doctor of Philosophy

in

Biology

by

Lauren Mack

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2016
The Dissertation of Lauren Mack is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

University of California, San Diego

2016
DEDICATION

To my sister, who has always been by my side. To my husband, for supporting me through my studies and has spent many weekends waiting for me to finish with my experiments.
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PUBLICATIONS


The role of Cryptochrome in Regulatory T cell development and function

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Doctor of Philosophy in Biology

University of California, San Diego, 2016

Professor Ye Zheng, Chair

Circadian rhythms are physiological processes that occur with a repeated period (in humans around 24 hours) that coordinate internal physiology with external stimuli. These changes are coordinated by a molecular transcriptional-translational feedback loop that includes high amplitude fluctuations in activity and expression of key molecular components. Several lines of evidence have indicated that the circadian clock may play a role in inflammation and immunity. A diurnal difference in the adaptive immune response has been observed
suggesting it is influenced by the circadian clock. However, studies have not yet been done about the circadian regulation of regulatory T cells (Tregs), which are an essential immune cell required for maintaining immune homeostasis. Tregs are a suppressive subset of effector CD4+ T cells expressing the transcription factor Foxp3, which are essential for preventing the development of autoimmune responses. Here we identified Cryptochrome (Cry), the main circadian transcriptional repressor, has important for Treg development and function. Cry deficiency in Tregs resulted in a marked competitive disadvantage in homeostatic proliferation compared to their wildtype Treg counterparts. This demonstrated that cryptochromes are required for optimal peripheral homeostasis of Tregs. Furthermore we identified Cry as important for Treg function; Cry deficient Tregs have compromised suppressive capacity both in vitro and in vivo. Mice with Cry deficient Tregs developed symptoms of immunopathology due to increased activation of CD4+ and CD8+ T cells as well as increased Interferon gamma (IFN-g) production by CD4+ T cells. RNA sequencing revealed the biological processes that were the most significantly altered in CryDKO Tregs; processes involved in rhythmic processes and mitochondrial respiration. Further analysis revealed that CryDKO Tregs had increased mitochondrial respiratory capacity resulting from increased mitochondrial membrane potential. These results have identified a novel component necessary for Treg cell homeostasis and reveals that there are many more complexities waiting to be discovered in Treg biology in regards to the role of the circadian clock and cellular metabolism.
Chapter 1: Introduction

1.1 Regulatory T cells

1.1.1 Overview of Regulatory T lymphocytes and Foxp3

Regulatory T cells (Tregs) are a suppressive subset of effector CD4+ T cells that are critical for maintaining immune homeostasis and tolerance to self-antigens; preventing development of autoimmune responses. Treg cell dysfunction contributes to the development of autoimmune disease and pathological tissue damage in both mice and humans, whereas overabundance of Tregs can promote chronic infection and tumorigenesis. Compromised Treg function is associated with several autoimmune diseases including type-1 diabetes, inflammatory bowel disease, and multiple sclerosis (Dominguez-Villar et al., 2011; Viglietta, 2004; Wildin et al., 2001). Tregs were originally defined by the high expression of CD25, the alpha subunit of Interleukin-2 (IL-2) receptor, but after the discovery of their essential transcription factor, forkhead box P3 (Foxp3), are now defined by expression of Foxp3 (Fontenot et al., 2003; Sakaguchi et al., 2011)

Foxp3, a member of the forkhead transcription factor family, is essential for the development and function of Tregs. Deletion of Foxp3 in mice (Scurfy) and humans (IPEX syndrome) leads to the development of lethal autoimmune disease in both resulting from splenomegaly and severe multi-organ
autoimmunity (Brunkow et al., 2001; Wildin et al., 2001). Foxp3 has been shown to be both a transcriptional activator and repressor greatly altering the gene expression profile of Tregs in comparison to conventional CD4 T cells (Tconv). Foxp3 is not only needed for the initial development of Tregs but continuous expression is necessary to maintain Treg function.

Besides Foxp3, many other factors contribute to Treg stability and function in vivo; including expression of a number of genes commonly referred to as Treg signature genes including Interleukin-2 receptor α chain (IL-2RA or CD25), cytotoxic T lymphocyte antigen 4 (CTLA4), tumor necrosis factor receptor superfamily member 18 (TNFRSF18 or GITR), and inducible T cell co-stimulator (ICOS). Foxp3 expression stability is also aided by expression of many other cofactors such as nuclear factor of activated T-cells (NFAT), forkhead box O3 (FOXO), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), signal transducer and activator of transcription 3 (STAT3), Gata-binding 3 (GATA3), and runt-related transcription factor 1 (RUNX1) (Feng et al., 2015; Kitoh et al., 2009; Li et al., 2014; Rudra et al., 2012). There have been other transcription factors identified as Foxp3 interacting partners or necessary for Foxp3 stability but will not be reviewed here. Foxp3 expression is stably maintained in vivo but under inflammatory conditions Tregs can their expression of Foxp3 showing that continuous maintenance of cellular processes in Tregs in necessary to maintain immune tolerance.
1.1.2 Regulatory T cell development and heterogeneity

Development of Tregs can occur in either the thymus or periphery, resulting in thymic derived Tregs (tTregs) or peripheral derived Tregs (pTregs) respectively. It is estimated that the majority of Tregs (70-90%) are tTregs while a smaller proportion are pTregs, which mostly reside in mucosal interfaces with the environment (Thornton et al., 2010; Weiss et al., 2012; Yadav et al., 2012). Both tTregs and pTregs have been shown to be important for maintaining immune homeostasis but develop and are activated under very different conditions.

Thymic derived Tregs (tTregs) are Foxp3 positive cells that arise in the thymus and require high affinity T cell receptor (TCR) ligation by agonist peptide-MHC complexes for development (Pacholczyk and Kern, 2008). T cells develop in two stages in the Thymus; first undergoing positive selection where cells that are not able to bind MHC complexes die by neglect followed by negative selection where T cells with high affinity TCR are deleted by apoptosis. tTreg development occurs at the second stage of T cell development. Continuous high level of TCR signaling in the Thymus can lead to deletion of thymocytes, however an alternative fate for the cells receiving intermediate TCR signaling is their development into Tregs by obtaining Foxp3 expression. This helps intermediate affinity T cells from developing into autoreactive T cells and maintains immune homeostasis. Supporting this theory, It was shown that Tregs in the thymus have more TCR activation than conventional CD4 T cells (Moran et al., 2011). The same study demonstrated that tTregs have higher levels of TCR signaling in the
periphery showing that they are regularly experiencing low-level self-antigen activation. After tTregs obtain Foxp3 expression and finish the maturation process in the thymus they migrate into the blood and secondary lymphoid organs.

Treg cells can also be generated from antigen stimulation in the periphery from mature conventional T cells and are known as peripheral Tregs (pTregs), generally localized at mucosal surfaces that interface with the environment. These cells can also be differentiated in in vitro assays, generating induced Tregs (iTregs), particularly in the presence of the anti-inflammatory cytokine transforming growth factor b (TGF-b). These pTregs have been shown to be important to redirect T cells that escaped negative selection in the Thymus and to maintain immune tolerance against non-pathogenic non-self antigens such as the antigen present in the microbiota and food products (Apostolou and Boehmer, 2004; Kretschmer et al., 2005). Both subsets of Tregs are primarily found in the secondary lymphoid tissues; the bone marrow, spleen and lymph nodes and circulating in the blood and lymph. However, recent studies have demonstrated their presence in a number of non-lymphoid tissues under resting conditions and have been indicated to have an affect on non-immunological processes (Sather et al., 2007). Tregs have been found in significant numbers in the skin, lungs, liver, intestinal mucosa, adipose tissue and placenta under non-inflammatory conditions (Burzyn et al., 2013).

Depending on the current expression of cell surface receptors Tregs can
be subdivided into two groups; activated and resting Tregs. This is based on the expression of T cell markers for activation and lymphoid homing. Resting or Central Tregs have high expression of CD62L in mice or CD45RA in humans, similar to naïve conventional T cells. Activated or effector Tregs have the opposite expression of these markers, low expression of CD62L or CD45RA as well as indications of recent encounters with antigen (Huehn et al., 2004; Miyara et al., 2009). Resting Tregs reside primarily in lymphoid tissues such as the spleen and lymph nodes while activated Tregs primarily reside in non-lymphoid tissues. Tregs that have recently left the thymus, are known as recent thymic emigrants (RLE), and have been shown to have a resting phenotype (Smigiel et al., 2014). Neither Treg type is locally maintained as both activated and resting Tregs are constantly recirculating though the lymph and blood (Luo et al., 2016). It has been seen that the maintenance of resting Tregs relies solely on IL-2 signaling while activated Tregs need both TCR stimulation and IL-2 (Levine et al., 2014; Vahl et al., 2014). In a recent study it was also demonstrated that activated Tregs live longer than resting Tregs (Luo et al., 2016). However, it is important to note that it is not known if these Treg subtypes are terminal differentiated into resting and activated or if these expression patterns seen are reversible. Furthermore, the functional and proliferative distinctions between activated and resting Tregs has yet to elucidated.
1.1.3 Mechanism of immunosuppression by Tregs

Tregs have been shown to essential for immune homeostasis and preventing immunodysfunction in both mice and humans but there is no one specific mechanism that is utilized by Tregs to suppress the immune response. Tregs suppress immune responses through multiple mechanisms that are tissue and context dependent and it seems multiple mechanisms of suppression are used simultaneously. Immunosuppresion by Tregs can be directed through disrupting effector T cell responses by cell-to-cell contact or deprivation of cytokines or metabolites, production of anti-inflammatory cytokine, modulating the activation and function of antigen presenting cells, and direct cytolysis of target cells (Vignali et al., 2008; J. B. Wing and Sakaguchi, 2012). Tregs express a number of cell surface receptors that have been implicated in suppression of effector T cell responses such as CTLA-4, which causes the downregulation of co-stimulatory molecules CD80 and CD86 in dendritic cells leading to less effective T cell priming (Qureshi et al., 2011; K. Wing et al., 2008). Another cell surface receptor highly expressed in Tregs is IL-2rα as known as CD25. CD25 is essential for Treg cell homeostasis but is also inhibits effector T cell proliferation by removing IL-2 from the local environment (Pandiyan et al., 2007). Other Treg cell surface receptors that have been shown to be important for Treg suppression include CD39, CD70, TIGIT, Neuropilin-1, GITR, OX40, and TNFRII. Tregs can also produce immunosuppressive cytokines such as IL-10, IL-35, IL-9, granzyme B and TGF-β but this mechanism of Treg suppression seems to be the most
important at mucosal surfaces that encounter the external environment (Rubtsov et al., 2008). It has also been shown that Tregs can cause direct cytolysis of either effector T cells or APCs that is mediated by the production of granzyme B (Cao et al., 2007; Gondek et al., 2005).

1.2 Metabolism in conventional and regulatory CD4+ T cells

1.2.1 Overview of cellular metabolic pathways

Emerging evidence demonstrates the importance of metabolic programs in T cell fate decisions and function. Changes in cellular metabolic programs have been primarily studied upon T cell activation; in which the rapid increase in energetic and biosynthetic demands are meet by rapidly altering cellular metabolism. It has also been shown that lymphocyte cellular metabolism can affect T cell differentiation and functional outcomes.

It is first important to overview the basic energetic pathways that are used by the cells to maintain consistent energy levels. Adenosine Triphosphate (ATP) is the principal molecule of energy for cellular processes. Maintaining energy levels is essential for cell survival and homeostasis of all cells. Two major metabolic processes that together efficiently convert glucose to ATP are glycolysis and oxidative phosphorylation (OXPHOS). Glycolysis is an oxygen independent cellular process converting glucose to pyruvate making reduced nicotinamide adenine dinucleotide (NADH) and ATP. Pyruvate is then converted to lactate at the same time regenerating NAD+. It generates byproducts that can
be further utilized for biosynthetic pathways but produces much less ATP per glucose molecule that oxidative phosphorylation. Oxidative Phosphorylation (OXPHOS) is an oxygen dependent process that produces much more ATP per glucose molecule by fully oxidizing pyruvate into CO$_2$ by generating a proton gradient across the mitochondrial inner membrane, which is used by ATP synthase to add a phosphate onto the metabolized ADP to regenerate ATP.

1.2.2 Cellular Metabolism and affect on T cell function

Activation of naïve T cells strongly enhances glucose uptake and induces a change in energetic pathways from oxidative phosphorylation to aerobic glycolysis (E. L. Pearce and E. J. Pearce, 2013). Thus occurs even in the presence of sufficient oxygen, a phenomena known as the Warburg affect (Vander Heiden et al., 2009; Warburg, 1956). Heightened aerobic glycolysis and glutaminolyis in activated T cells supports ATP generation but more importantly provides the building blocks for biosynthesis of amino acids, nucleotides, and lipids. Functionally distinct T cell subsets require distinct energetic and biosynthetic pathways to support their functional needs.

The general consensus of cellular energetic pathways in T cells is that effector T cells utilize glycolysis while naïve and memory T cells utilize oxidative phosphorylation (E. L. Pearce et al., 2009). However, this paradigm does not always hold true both in vitro and in vivo (Blagih et al., 2015; Byersdorfer et al., 2013; Gatza et al., 2011). In environments deplete of specific nutrients, effector T cells have been shown to be capable of using OXPHOS for energy production.
(Blagih et al., 2015). It was also seen that during graft-versus-host disease alloreactive T cells increase levels of OXPHOS specifically fatty acid oxidation (FAO) (Chiaranunt et al., 2015).

It is now evident that metabolic programs are intimately linked to T cell activation and function. It is important to understand the tuning of these programs during different stages of T cell life and how it could be manipulated for novel therapeutics. Shortly upon activation T cells prefer glycolysis for energy production, which is aided by increasing uptake of glucose and glutamine while down regulating FAO (Chang et al., 2013; Wang et al., 2011). This metabolic reprogramming has been determined to be essential for naïve CD4 T cells to differentiate into distinct effector T cell lineages and to be fully capable of making effector molecules (Chang et al., 2013; Mascanfroni et al., 2015; O'Sullivan et al., 2014; Sukumar et al., 2013; van der Windt et al., 2012; Yin et al., 2015). Failure of T cells to upregulate glucose metabolism upon T cell receptor activation results in decreased cytokine production as well as proliferation terminating in apoptosis or anergy. However, T cell metabolic reprogramming can be influenced by strength of stimulation, antigen exposure time, and co-stimulation. It has been shown that metabolic programming alone can affect function of T cells while leaving differentiation status of those cells unaffected. Graft-versus-Host disease-model derived antigen-specific T cells and immunization-derived antigen-specific T cells have different metabolic profiles, relying more on OXPHOS, while
maintaining the same differentiation status of CD44hiCD62Llo (Byersdorfer et al., 2013)

1.2.3 Cellular Metabolism and Treg development and function

As discussed previously, Tregs can develop in two locations; in the thymus, generating tTregs or in the periphery generating pTregs, which can be generated in vitro and are known as induced Tregs (iTregs). Cellular metabolism in Tregs has primarily been studied in in vitro differentiated Tregs but the metabolic profile of Tregs in vivo as not been investigated thoroughly. First, the recent research on in vitro differentiated Tregs will be discussed followed by the studies that have been done to investigate the importance of cellular metabolic processes in vivo.

Since Tregs in the periphery develop from naïve T cells, the environmental cues that are derived from the available nutrients and hormones play an important role on the generation and survival of iTregs instead of generating other helper T cell (Th) subsets. Previous studies indicate that FAO is required for in vitro Treg generation and proliferation but the importance of oxidative phosphorylation in vivo as yet to be elucidated (Macintyre et al., 2014; Michalek et al., 2011; Pollizzi and Powell, 2014; Shi et al., 2011). In addition, It has been reported that compared to other helper T cells, iTregs have a unique metabolic profile of uniquely low levels of glycolysis and lower mechanistic target of rapamycin (mTOR) activity. In vitro nutrient conditions that favor fatty acid oxidation favors iTreg generation and iTregs have higher levels of FAO
compared to other Th cell subsets (Michalek et al., 2011). Another study showed that Acetyl-CoA carboxylase (ACC1) deficient CD4 T cells had increased Treg generation as well as AMP-activated protein kinase (AMPK) activity, which is correlated with increased FAO (Berod et al., 2014). In agreement with the hypothesis that Tregs rely primarily on FAO, multiple studies have shown that unlike other Th cell subsets, Treg generation increases with the inhibition of metabolic pathways that promote glycolysis. mTOR is a nutrient sensor that balances energy metabolism by means of the transcriptional control of glycolysis and mitochondrial oxidative function. One study demonstrated that iTreg generation is increased with the addition of rapamycin, which acutely inhibits mTOR activity (Delgoffe et al., 2011). Inhibition of mTOR signaling through the deletion of Hypoxia-inducible factor 1α (HIF-1α) also resulted in increased iTreg generation (Shi et al., 2011). The mTOR signaling pathway results in the upregulation of glycolysis and the increased Treg generation in its absence suggests that unlike other Th helper subsets iTregs are less dependent on glycolysis and their differentiation may even be promoted by its inhibition.

A few studies have suggested that iTregs may not be completely independent of a reliance on glycolysis, showing that in human iTreg generation glycolysis is necessary for their differentiation and survival through importance of the glycolytic enzyme enolase-1 (De Rosa et al., 2015). Another study has indicated that FAO inhibition had no affect on Treg numbers or frequency in vivo demonstrating that FAO may not be essential for Treg survival (Byersdorfer et al.,
This suggests that multiple cellular energetic processes may contribute to the function and generation of iTregs at least in human T cells. To enhance our understanding of Treg cellular metabolism it is necessary to study Tregs in vivo. The studies that have been done to analyze in vivo Tregs have suggested that the cellular metabolic profile of T cells may be more complex than has been previously suggested. Recent proteomic studies of human conventional and regulatory T cells have enhanced our understanding of the metabolic profile of both (Procaccini et al., 2016). Proteomic analysis suggests that Ex vivo activated Tregs rely on both glycolysis and FAO while conventional T cells primarily use glucose metabolism. This data suggests that the reason why Tregs are capable of surviving inhibition of glucose uptake or glycolysis is due to the fact that another bioenergetic pathway can sustain cell growth. Inhibition of either glycolysis or FAO affected Treg function and maintenance suggesting that both pathways are important for proper Treg homeostasis.

The affect of cellular metabolism on Treg stability and function is important to investigate to better understand the interplay between cellular metabolism and T cell function. There have been few studies that directly investigate the role of cellular metabolism in Treg function and stability. While Treg generation is enhanced by inhibition of mTOR, it is also necessary to maintain the suppressive capacity of Tregs (Zeng et al., 2013). Deletion of TORC1 activity in vivo leads to the development of early onset inflammatory disorder. This defect was shown to be caused by the role of mTOR signaling to promote cholesterol and lipid
metabolism important for Treg lineage specific markers. It was also demonstrated that mTOR signaling was important for Treg proliferation. This suggests that in accordance with proteomic studies, glycolytic pathways may be important for Treg proliferation \textit{in vivo} and may differ from the role that mTOR plays \textit{in vitro} for Treg generation.

PTEN is an upstream inhibitor of PI3K and can affect cellular metabolic pathways. It was seen that PTEN deficient Tregs have reduced expression of CD25 and rapidly lose Foxp3 expression (Shrestha et al., 2015). PTEN deficiency in Tregs also lead to increased glycolysis and reduction of mitochondrial mass and membrane potential suggesting that importance of PTEN in Treg stability may be through its affect on Treg metabolic programs.

Autophagy is a process in which cells degrade intracellular substrates in the lysosome for recycling. A recent study showed that there is more autophagosomes in Tregs than conventional T cells and that autophagy in Tregs was important for maintenance \textit{in vivo} (Wei et al., 2016). Mice deficient in Tregs capable of autophagy developed autoimmune disease due to increased rates of apoptosis and Interferon-gamma (IFNg) production by these cells. Autophagy in Tregs partial functions by restraining aberrant mTOR signaling thus inhibiting C-Myc expression and glycolysis. While these studies suggest a negative role for glycolysis in Treg cell stability and function other studies overviewed previously have suggested that glycolysis may be important in other contexts. There is
much to be discovered in the role of metabolism on Treg function and maintenance.

1.3 The Circadian clock

1.3.1 Overview of the circadian clock

Circadian rhythms are physiological processes that occur with a repeated period (in humans around 24 hours) that coordinate internal physiology with external stimuli. These rhythms are found in most prokaryotes and eukaryotes. The principal circadian pacemaker in mammals is the suprachiasmatic nucleus (SCN), a small region of the hypothalamus of the brain that consists of only 100,000 cells in humans (Moore and Card, 1985). In mammals, light activates a specific group of photoreceptors in the retina connected to the SCN which controls behavior and coordinates daily programs of gene expression across the body (Hastings et al., 2003). Non-light cues can also provide input to the clock from the external environment such as food, social interaction, and food (Mrosovsky, 1996). In the absence of external timing cues, the SCN is able to maintain synchronicity for weeks, which explains why animals housed in constant darkness maintain rhythmic behavior.

Although the SCN functions as the master circadian oscillator, reports demonstrate that peripheral clocks are crucial for maintaining local physiology (Schibler, 2006). After discovery of the circadian core clock genes it was shown
that clock genes are expressed as housekeeper genes and are expressed in almost every cell type (Lowrey and Takahashi, 2004). Both in the brain and peripheral organs circadian clock rhythmic expression is maintained in a cell autonomous manner, which controls oscillation of the specific clock gene outputs of the cell and can function independently of the SCN. These cell autonomous circadian rhythms are essential to maintain temporal organization of key processes such as metabolism and detoxification. Orchestration of the circadian clock occurs by circadian clock transcriptional translation feedback loops; which generates a feedback loop of transcriptional activation of repression to generate oscillations in clock output expression levels (Harmer et al., 2001).

The molecular mechanism of the circadian clock is comprised of an autoregulatory transcriptional network which includes high-amplitude fluctuations in activity and expression of key molecular components (L. Zhang et al., 2011). At the core, the transcriptional regulators brain and muscle ARNT-like protein 1 (BMAL1) and circadian locomotor output cycles kaput (CLOCK) act as a heterodimer transcription factor and activate transcription of clock-controlled genes (CCGs) by binding to E box enhancer elements in their promoter region (Gekakis et al., 1998). Transcriptional initiation includes transcription of the repressors period (Per1, Per2, and Per3) and cryptochrome (Cry1 and Cry2) genes. As the RNA and protein levels of these repressors increase they assemble into heterodimers, translocate to the nucleus, and repress CLOCK:BMAL1 mediated transcription. As Per and Cry represses
CLOCK:BMAL1 mediated transcription, Per and Cry expression also decreases and CLOCK:BMAL transcription resumes. This is coupled with subsequent post-translational modifications such as phosphorylation and ubiquitination which leads to the degradation of Per and Cry (Hirano et al., 2013). Since the discovery of the core circadian clock, supplementary feedback loops have been discovered that affect Clock:BMAL1 mediated transcription of clock related genes such as repression of BMAL1 by REV-ERBα (Preitner et al., 2002). Briefly, CLOCK:BMAL1 drive transcription of REV-ERBα and REV-ERBβ which competes with the ROR-PGC-1α complex for RORE elements within the CLOCK and BMAL1 genes and repress their transcription. Along with transcriptional repression and activation there are a number of post-translational modifications of these proteins that contribute to the homeostasis of the circadian clock including protein phosphorylation, ubiquitination, sumoylation, and acetylation. Additional phases are also generated by clock output regulators, which make an increasingly complex temporal transcriptional network. Studies have indicated that at least 10% of all transcripts are regulated in a circadian manner in tissues and this percentage increases to close to 20% of genes involved in metabolic processes (Eckel-Mahan et al., 2012; Minami et al., 2009; Storch et al., 2002).

There have been numerous studies that have demonstrated a link between cellular metabolism and activity of the circadian clock in a bidirectional relationship. The association of the circadian clock and metabolism occurs at both the cellular and physiological level. Peripheral circadian clocks have a major...
purpose in tissues to control metabolic processes, which fluctuate with the temporal consumption of food. A large number of metabolites and key metabolic functions have been found to oscillate over the course of the day including carbohydrate and lipid metabolism.

The circadian clock-metabolic interaction has been shown to affect many cellular processes. Circadian control of NAD production demonstrates the link between the circadian clock and mitochondrial function and then in turn regulates expression of circadian genes by the abundance of NAD and ATP. One example of the link between cellular metabolism and the circadian clock was shown in which Hepatic NAD+ levels oscillate during the day and is driven partial by the circadian expression of nicotinamide phosphoribosyltransferase (NAMPT) (Ramsey et al., 2009). It was also shown that in the liver changes in NAD+ levels through activation of SIRT3 controls daily changes in oxidative phosphorylation in the mitochondria (Peek et al., 2013). The circadian clock has been shown to be able to modulate mitochondrial function by protein acetylation demonstrating the close association of the circadian clock and cellular metabolism (Masri et al., 2013). The circadian clock-metabolic axis has been shown to play a role in the regulation of xenobiotic metabolism, cholesterol and fatty acid synthesis, translation, carbon metabolism, and cell cycles (Brown:2016dl).

It is important that the central and peripheral circadian clock are synced, there is mounting research into the circadian clock that has demonstrated the importance of clock homeostasis and human health. Disruption of the circadian
clock, either by chronic jet lag or shift work, has been associated with various diseases including metabolic syndrome, mental health disorders, numerous cancer incidences, and susceptibility to infections (Ferrell and Chiang, 2015). One short-term well-controlled study demonstrated that shifting the time of sleep alone without altering the duration of sleep in healthy volunteers increased inflammation and insulin insensitivity acutely. This study demonstrates that even short-term disruption of the circadian clock can lead to unfavorable physiological changes (Leproult et al., 2014).

1.3.2 Circadian clock and the immune system

Several lines of evidence have indicated that the circadian clock may play a role in inflammation and immunity. Several inflammatory diseases vary in severity over the course of the day including bronchial asthma and rheumatoid arthritis (Durrington et al., 2014; Gibbs and Ray, 2013). Disruption of circadian rhythm in both animals and humans by chronic jet lag or shift works as been association with immune system dysfunction and a higher rate of immunopathology (Castanon-Cervantes et al., 2010; Knutsson, 2003).

Variation of the immune system’s response to infections throughout the day was noted in the past, the survival rate of mice varies depended on the hour of bacterial infection (Bellet et al., 2013; Feigin et al., 1969; Wongwiwat et al., 1972). In mice, variation of the immune cell response throughout the day suggests that there is a biological rhythm of immune responsiveness, it appears that immune alertness fades during the resting phase (daytime in mice) when the
animals are less likely to encounter pathogens (Scheiermann et al., 2013). Susceptibility to infection is elevated in the late rest phase and during transition to the active phase. Also, whole blood stimulation with lipopolysaccharide (LPS) resulted in less inflammatory cytokine production in the early morning compared to early night in healthy donors. Leucocyte migration is also affected by diurnal variations; cell numbers oscillate throughout the day. Many immune cells numbers are lower in the morning than the rest of the day, which promotes the idea that during the resting period immunological memory is formed and then migrate to ensure responsiveness in localized environments during the active period (Abo et al., 1981). Supporting this theory, deletion of the circadian clock in monocytes eliminated the variation in numbers seen in monocyte populations during the day and leads to increased inflammation in tissues (Nguyen et al., 2013). Circadian control of the immune system could originate from the circadian rhythm of either of both the central clock of the SCN or from processes controlled by a cell-autonomous circadian clock.

Much of the current research in the immune-circadian interface focuses on identifying a cell autonomous clock in immune cells and investigating what immune cellular processes are under circadian regulation. Recent evidence demonstrates a cell-autonomous circadian clock in numerous immune cell subsets including macrophages, dendritic cells, natural killer cells, as well as T and B cells (Arjona and Sarkar, 2005; Bollinger et al., 2011; Keller et al., 2009; Silver et al., 2012a). A functional circadian clock in the immune system has been
shown to directly regulate a number of immune function such as regulation of proinflammatory cytokines and pattern recognition receptor recruitment of immune cells to tissues, antigen presentation and lymphocyte proliferation (Geiger et al., 2015; Gibbs et al., 2012; Narasimamurthy et al., 2012; Scheiermann et al., 2013; Silver et al., 2012b).

### 1.3.3 Circadian clock in T Cells

Diurnal differences in the adaptive immune response and variation of total lymphocyte numbers has been observed suggesting that the adaptive immune system is also under circadian control. Variation of lymphocyte numbers in the blood in humans leads to the highest concentration of T cells in the late evening and declines throughout the day although no variation in lymphocyte numbers are seen in the lymph nodes. This has been attributed to the levels of cortisol in the blood and increased expression CXCR4, a chemokine receptor, in T cells (Besedovsky et al., 2014). It has been shown that both antibody responses and responses to vaccination have measurable diurnal variation (Fernandes et al., 1976; Silver et al., 2012b). The magnitude of both T cell and B cell responsiveness to immunization depends on the time of administration suggesting that the central clock and/or lymphocyte autonomous clocks could have an affect of lymphocyte function (Fortier et al., 2011).

Selected studies have evaluated whether T and B lymphocytes express the different clock genes and if their expression is rhythmic and important for lymphocyte function. Lymph nodes have several clock genes that are
rhythmically expressed and this rhythm is attenuated in CLOCK mutant mice
(Fortier et al., 2011; Keller et al., 2009). CD4 T cells have been shown to have a
rhythmic expression of Per2 in cells isolated from the spleen and the thymus,
which indicates that CD4 T cells have expression of a cell intrinsic circadian clock
(Bollinger et al., 2011). Similar, human CD4 T cells from blood have variation of
expression of several clock genes over a 24 hour time period.

It has been indicated that both CD4 and CD8 T cell cytokine response
and proliferation to TCR activation is stronger during the active phase then
resting phase (Bollinger et al., 2011; Fortier et al., 2011). Recently it was also
shown that there is diurnal regulation over differentiation into IL-17-producing
CD4 helper T cells (Th17) and is affected by disrupting the circadian clock with a
model of chronic jet-lag/time shifting (Yu et al., 2013). However, these studies do
not address if the importance of the circadian clock is from the role of the cell
intrinsic clock in T cells or caused by whole body changes emanating from the
central circadian clock. One recent study has tried to address this question by
disrupting the circadian clock by deletion of BMAL1 specifically in T lymphocytes
(Hemmers and Rudensky, 2015). The proportion of IL-2 production by T cells
after bacterial infection did vary depending on the time of infection but was
independent of the cell intrinsic circadian clock since deletion of BMAL1 had no
affect on IL-2 production. It was also seen that BMAL1 deficiency had no affect
on T cell differentiation or function except in a competitive setting where a slight
deccrease in IL-2 production was observed. This study suggests that the cell
intrinsic clock may not be as important for T cell function as the central clock. Nevertheless, more studies are needed to investigate since there are several interlocking loops and outputs of the circadian clock that could cause a different result.

1.4 Cryptochromes

1.4.1 Cryptochrome discovery and background

Cryptochrome was first discovered as a key regulator of the circadian molecular clock. As mentioned earlier, Cryptochromes repress CLOCK:BMAL1 transcription of core clock genes when localized in the nucleus. However, the exact molecular mechanism of repression has yet to be completely elucidated and much remains to be determined about the biochemical interactions of Cry with other proteins. Cryptochromes are thought to have developed from photolyases, bacterial light-activated DNA repair enzymes, and are still quite similar structurally; with an N-terminal α/β domain and a C-terminal α helical domain that is a flavin-binding site (Sancar, 2003). These structural attributes and similarity to plant cryptochromes is what lead to their initial discovery in animals. Cryptochromes are recruited to thousands of sites outside the core clock related genes driven by CLOCK:BMAL1 transcription demonstrating that they have unique affects on molecular pathways outside of the circadian transcriptional-translation-negative feedback loop (Koike et al., 2012). However, overexpression of CRY1 protein does not lead to period alteration (Chen et al.,
2009). When Cry1 and Cry2 knockout mice were generated it was seen that these mice had disrupted circadian rhythmicity with shorter or longer periods, respectively (van der Horst et al., 1999; Vitaterna et al., 1999). If both Cry1 and Cry2 are deleted the double knockout mouse loses all circadian rhythmic activity and cyclic clock gene expression when in constant darkness. The differential affect of the deletion of Cry1 and Cry2 on rhythmic activity might be explained by differential transcriptional expression patterns as well as different post-translational modifications such as ubiquitylation, phosphorylation, and acetylation.

1.4.2 Biological processes affected by Cryptochrome

Cryptochrome (Cry) takes part in the core molecular circadian clock acted as the primary repressor of CLOCK:BMAL1 mediated transcription of the core clock genes. It is important to note that only BMAL1 and Cry1/2 deficient mice have a complete and immediate loss of circadian rhythms demonstrating their non-redundant role in the maintenance of the circadian clock (Bunger et al., 2000; van der Horst et al., 1999). Cry stability has an essential role in the determination of the circadian period. The levels of Cry expression are affected by many post-translational modifications, which then lead to disruption of the circadian period. Two proteins involved in the stability of Cry, by mediating its ubiquitination, are F-Box And Leucine-Rich Repeat Protein 21 (FBXL21) and F-Box And Leucine-Rich Repeat Protein 3 (FBXL3) (Hirano et al., 2013; Siepka et al., 2007; Yoo et al., 2013). When either FBXL3 or FBXL21 are mutated there is
a robust alteration of the circadian clock. It was also seen that phosphorylation of Cry1 by AMPK increases its association with FBXL3 interrupting its interaction with Per and leading to its degradation (Lamia et al., 2009; Xing et al., 2013). This shows that the Cry expression and stability is essential for maintenance of the circadian molecular clock.

Cry is important for the maintenance of the circadian clock but also has an affect on a number of other biological processes, identifying it as an output molecule for the circadian clock. Consistent with this idea Cry1 and Cry2 have been found to each bind thousands of sites in the genome outside of those bound by the clock transcription factors which gives evidence that Cry proteins are promiscuous transcriptional repressors that can affect many biological pathways (Koike et al., 2012).

As previously mentioned the fact that AMPK phosphorylation can lead to Cry degradation shows that the circadian clock can be affected by changes to the energetics of the cell. It has also been shown that Cry can affect cellular pathways within the cell. Interestingly, genetic studies in humans have indicated that variants in Cry1 as well as MTNR1B (Melatonin) genes are associated with glucose levels in humans suggesting a link between Cry expression and glucose homeostasis and metabolic disease (Dupuis et al., 2010; Lyssenko et al., 2009; Prokopenko et al., 2009). In agreement, Mice deficient in Cry are more susceptible to obesity when challenged with a high fat diet even while displaying hypophagia and although they show slightly decreased body weight under
normal chow conditions (Barclay et al., 2013; Bur et al., 2009). Cry deficient mice exposed to high fat diet also had hyperinsulinemia due to potentiated insulin secretion which correlated with increased lipid uptake of insulin. The exact mechanism of Cry on glucose homeostasis has been investigated and this research has shown a direct link between Cry expression and glucose metabolism. In mice, Cry deficiency results in glucose intolerance and high levels of corticosterone as well as increased susceptibility to glucocorticoid induced hyperglycemia (Lamia et al., 2011). This is due to the fact that normally Cry1 and Cry2 repress the activity of glucocorticoid receptor by associating with a glucocorticoid response element in the phosphoenolpyruvate carboxykinase 1 (pck1) promoter.

Cry also seems to have an effect on the accumulation of cAMP within a cell affecting gluconeogenesis pathways. It was seen that Cry Proteins directly inhibit G protein signaling decreasing gluconeogenesis in the liver and affected glucose homeostasis during the fasting (rest) period of mice, when Cry is most highly expressed (E. E. Zhang et al., 2010). This study described a novel mechanism of Cry; by directly interacting with GαS Subunit of the G-coupled protein receptor directly in the cytosol affecting the accumulation of cAMP and downstream PKA mediated activation of CRE-responsive element binding protein (CREB). This suggests that there may be effects of Cry proteins with substrates outside of the transcriptional regulation that they exert. In accordance with this study it was also seen that a drug, KL001, which stabilizes Cry, inhibited
glucagon-induced gluconeogenesis in primary hepatocytes (Hirota et al., 2012).

Cry deficiency has also been shown to increase oxidative phosphorylation in mouse embryonic fibroblasts while the opposite was seen when cells were deficient in BMAL (Peek et al., 2013). All these data demonstrate the Cry can have a direct affect on cellular metabolic pathways.

Cry deficiency has also been shown to affect many other physiological processes. Cry deficiency also increases the susceptibility to salt induced blood pressure elevation due to the fact that they have constitutively elevated levels of aldosterone (Doi et al., 2010). Cry proteins also protect genome integrity through coordination of downstream transcriptional response to DNA damage (Papp et al., 2015). This data suggests that Cry may be an important for protecting cells undergoing cell division while undergoing DNA replication.

There have been some studies that suggest that Cry may be important in immune cells and for the prevention of autoimmune disease. Cry deficient mice develop more severe induced arthritis characterized by increased joint swelling and increases production of the proinflammatory cytokines TNF-a, IL-1b and IL-6, and matrix metalloproteinase-3 (Hashiramoto et al., 2010). Also, deletion of Cry increases constitutive expression of proinflammatory cytokines in macrophages in a cell autonomous manner (Narasimamurthy et al., 2012). Absence of Cry lead to increased NF-κB expression due to the absence of Cry mediated repression of GPCR signaling. All these studies highlight the role in Cry in both the circadian clock as well as other important molecular pathways.
As our understanding of both Treg biology and the circadian clock increases it is important to ask the question if there is any interaction between the two. There as of yet has not been many studies attempting to understand the role of circadian clock genes in T cells and none that have attempted to investigate their role in Tregs. In my research I attempted to address this question by investigating the potential role of the main circadian repressor, Cry, on the development and function of Tregs in the hopes to better understand Treg cell biology. Our studies have identified Cry has a novel molecule important for the regulation of both Treg development and function. In the following chapters I will describe my findings on the function of Cry in Treg biology. In chapter 2 I will describe the material and methods that were used to do the research for my thesis work; in chapter 3, I will discuss the finding of the role of Cry in Treg cell development and proliferation; in chapter 4, I will address the role of Cry in Treg function; in chapter 5, I will examine the molecular pathways that may be affected by Cry deficiency in Tregs; finally in chapter 6 I will summarize our data and discuss further research to be done and the implications of our findings.
Chapter 2: Materials and Methods

2.1 Mice

All mice were housed in the specific pathogen-free facilities at The Salk Institute for Biological Studies or purchased from the Jackson Laboratory. All procedures involving animals were performed in accordance with protocols approved by the IACUC and Animal resources Department (ARD) of the Salk Institute for Biological Studies. Cry1 KO and Cry2 KO mice were provided from Ronald Evans laboratory at the Salk Institute for Biological Studies (Lamia et al., 2009). Cry 1 -/+ and Cry2 -/+ heterozygous mice were crossed with Foxp3 Thy1.1 (Liston et al., 2008) reporter mice to utilize for isolation of Tregs from the spleen and lymph node. Cry 1 -/+ Foxp3 Thy1.1 +/+ and Cry2 -/+ Foxp3 Thy1.1 +/+ heterozygous mice were further crossed to produce Cry double KO mice for analysis and further experiments.

2.2 Generation of Bone Marrow Chimeras

Bone marrow (BM) chimeric mice were generated from single suspensions of BM extracted from the femurs of indicated genotypes. Foxp3 KO BM was first depleted of T cells with Thy1.2 antibody coated magnetic beads according to the manufacturers instructions (Mouse Pan T cell Dyanbeads, ThermoFisher). BM cells were transferred individually or at a 1:1 ratio into lethally irradiated (1000rad) Rag1 KO recipients (2.5 x 10^6 total cells/recipient). BM engraftment
was analyzed in blood by flow-cytometric analysis at the indicated time periods. Mice were euthanized for analysis of immune cell composition in spleen, lymph node and thymus; and for analysis of organ tissue histology and clinical symptoms.

2.3 Histological Analysis

Tissues were fixed in 4% phosphate-buffered formalin before cut into sections. Sections were stained with haematoxylin and eosin according to standard procedures. A pathologist graded histopathology on blinded samples for severity and extent of inflammation and morphological changes.

2.4 IL-2 Expansion of Tregs

IL-2-anti-IL-2 Complexes were prepared by incubating 2ug of murine IL-2 (Biolegend) with 10ug of anti-mIL-2 antibody (Jes6.1, BioXCell) in 200 μL of phosphate buffered saline (PBS) per injection for 30 minutes at 37°C. Mice were given an intraperitoneal (i.p) injection for three sequential days and analyzed on day 6 after first injection.

2.5 Treg cell isolation and adoptive transfer

Total CD4+ T cells were isolated from the spleen and lymph nodes using the Dynabeads CD4 positive isolation kit according to the manufacturers instructions (ThermoFisher). Tregs were then isolated by either FACs sorting (Foxp3Thy1.1+) or using anti-PE magnetic bead isolation (Miltenyi) using Thy1.1 PE (Ebioscience Clone OX-7 (H1SS1)). Naïve T cell responder cells were
isolated by first isolating total CD4+ T cells as described above and further purified by sorting on CD4+CD25-CD62L-CD44hi T cell population. Treg adoptive transfer was performed by injection of 0.25 x10^6 WT and CryDKO Tregs cells along with 0.5 x10^6 naïve T responder cells into each Rag1KO recipient mouse and analyzed 10 days after transfer for Treg frequency.

2.6 In Vivo Treg Proliferation Assay

0.5 x10^6 WT or CryDKO Tregs cells were transferred by retro-orbital injection along with 0.5 x10^6 naïve T responder cells in each recipient Rag1KO mouse and analyzed 14 days later for frequency and absolute number of transferred cells in the spleen, lymph nodes and mesenteric lymph nodes.

2.7 In Vivo T cell induced colitis

CD4+Foxp3-CD45RBhi T cells were isolated by FACs sorting. 0.5 x10^6 CD4+Foxp3-CD45RBhi T cells were transferred into Rag1KO recipient mice alone or 0.1 x10^6 WT or CryDKO Tregs. Weight was monitored over the course of the experiment on a weekly basis. At the end of the study the mice were euthanized for FACs analysis of lymph nodes and spleen.

2.8 Flow Cytometry Staining

Single cell suspensions of lymphoid organs were obtained and stained with antibodies for cell surface receptors. The following antibodies were used for cell surface staining: CD45.1(A20), CD45.2 (104), CD8 (53-6.7), CD4 (GK1.5), Thy1.1 (OX-7), B220 (RA3-6B2), CD44 (IM7), CD62L (MEL-14), CD25 (PC61),
CD3a, TCRβ (H57-597), CD3ξ (145-2C11). Foxp3 (FJK-16s) staining was done using Foxp3 transcription factor staining buffer set (Ebioscience) according to the manufacturer’s instructions. Cytokine production was measured after stimulation with PMA (phorbol 12-myristate 13-acetate; 50ng/ml; Sigma) and ionomycin (1μg/mL; Sigma) in the presence of Brefaldin A (Golgiplug™, BD biosicences) for 5 hours at 37°C before intracellular staining for cytokines. Intracellular staining were then fixed and permeabilized (Ebioscience) and labeled with appropriate antibodies before being analyzed on BD FACS ARIAII. Antibodies used for cytokine intracellular analysis were IFNγ (XMG1.2) and IL-4 (BVD6-24G2). Results were analyzed with Flowjo (TreeStar).

2.9 In Vitro Suppression Assay

Tregs were isolated from WT and CryDKO mice as explained above. CD45.1+ congenic mice were use for isolation of CD4+CD25-CD62LloCD44hi responder T cells. Antigen-Presenting Cells (APC) were prepared from congenic CD45.1+ B6 splenocytes by T cell depletion using Thy1-specific Dynabeads kit (ThermoFisher) and irradiated (25 Gy). CFSE (5μM, Invitrogen) labeled responder T cells (5 x 10⁴) were cultured with irradiated APCS (1x10⁴) and indicated ratio of Tregs in 96 well plates in Complete RPMI-1640 medium supplemented with 5% Fetal Bovine Serum (FBS) and anti-CD3 antibody (1μg/mL). Cell proliferation was determined 96 hours later by FACS analysis.
2.10 Retroviral Infection of Tregs

HEK 293T cells were seeded at $0.4 \times 10^6$ cells per well in a 6 well plate the day before transfection. 2 $\mu$g total plasmid DNA was transfected using Fu-GENE 6 (Promega) containing pCL-Eco (Addgene plasmid 12371) and retroviral expression plasmid. Viral supernatant was collected 48 and 72 hours after transfection. Tregs were stimulated for 1 day with plate coated Goat-Hamster IgG antibody (200ng/ml; MP Biomedicals) and soluble anti-CD3 (1mg/ml; 145-2C11), anti-CD28 (1mg/ml; 37.51) (Bio X Cell), and hIL-2 (500U/ml; Tonbo) before spin infection with viral supernatants with 4 $\mu$g/ml polybrene (Millipore) for 90 minutes 2500rpm at 32°C. Tregs were collected and FACs sorted for GFP+ cells 4 days after activation and used for further assays.

2.11 In Vitro Treg culture

All Tregs used for in vitro culture were isolated from WT or CryDKO BM chimera mice to avoid extrinsic affects of gene disruption on cells. Tregs were stimulated for 3 days with plate coated Goat-Hamster IgG antibody (200ng/ml; MP Biomedicals) and soluble anti-CD3 (1mg/ml; 145-2C11) and anti-CD28 (1mg/ml; 37.51) (Bio X Cell) and hIL-2 (500U/ml; Tonbo) in complete RPMI1640 medium. Live cells were isolated by Ficoll-Paque Premium (GE life sciences) for downstream assays.
2.12 Metabolic Assays

OCR and ECAR were measured with an XFe96 extracellular flux analyzer (Agilent Technologies). In vitro activated Treg cells were plated in equal numbers in XF plates for analysis using Cell-Tak™ (BD Bioscience). Measurements were taken in unbuffered RPMI (Gibco). For reading, OCR was measured over time following injection of oligomycin (0.5μM), Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) (1μM), Rotenone (0.75μM) and Antimycin A (0.75μM) all from Sigma.

2.13 Mitochondrial Assays

Mitochondrial content was measured by the ratio of mitochondrial DNA to genomic DNA. Total DNA was isolated from cell lysate of In Vitro activated Tregs by Phenol:Chloroform Extraction (ThermoFisher). Total DNA concentrations were measured using a Nanodrop and concentrations were normalized between samples. Relative quantity of cytochrome c oxidase subunit I (CO1) and NDUFV1 was measured by qPCR amplification using SYBR Green PCR Master Mix (Applied Biosystems). Quantitative PCR was performed on an Applied Biosystems ViiA™ 7 Real-Time PCR System. The CO1 primers were 5'-TGCTAGCCGCAGGCATTA C-3' (forward primer) and 5'-GGGTGCCCAAAGAATCAGAAC-3' (reverse primer). The NDUFV1 primers were 5'-CTTCCCCACTGGCCTCAA G-3' (forward primer) and 5'-CCA AAA CCC AGT GAT CCA GC-3'
(reverse primer). Reactive oxygen species production was measured by incubating In Vitro activated Tregs with 2.5 μM CM-H₂CFDA (ThermoFisher) for 30 minutes in warmed DMEM (Gibco) Medium followed by FACs staining. Mitochondrial membrane potential was measured using the Mitoprobe™ DiOC₂(3) Assay kit (ThermoFisher) according to the manufacturer’s instructions for FACs analysis.

2.14 cAMP Assays

In Vitro activated Tregs were used to measure cAMP either without drug treatment or by pre-incubation with Forskolin (20μM, Sigma) or PGE2 (1μM, Sigma) for 30 minutes in warmed DMEM media before cell lysis. cAMP was measured using the cAMP femto HTRF kit (Cisbio) according to manufacturer’s instructions. 3′,5′-hydrogen phosphorothioate adenosine triethylammonium salt, Rp-cAMPS (100ug/mL Sigma) was added to Treg in vitro suppression assay twice daily.

2.15 RNA Sequencing

Tregs cells were FACs sorted after co-culturing with Naïve T cell responder cells and irradiated APCs with soluble anti-CD3 (1mg/ml) for 96 hours. RNA was extracted with TRIzol reagent (Life Technologies). Sequencing libraries were prepared from 10–100 ng of total RNA using the TruSeq RNA sample preparation kit v2 (Illumina). RNA-Seq libraries prepared from two biological replicates for each experimental condition were sequenced on the
Illumina HiSeq 2500 using barcoded multiplexing and a 100-bp read length. Reads were aligned to the mouse genome (mm9, NCBI37) using STAR. Gene expression values were generating for RefSeq annotated transcripts using Gene Ontology biological processes and clustering analysis was performed using Metascape (http://metascape.org) (Tripathi et al., 2015).

2.16 Statistical Analysis

The sample size (n) for each experimental group is described in each figure legend. Graphpad Prism was used for all statistical analyses. Data displayed in histograms are expressed as means ± standard deviation (error bars). p values were calculated using two-tailed unpaired Student’s t test for comparisons between two groups or two-way analysis of variance (ANOVA). p values of less than 0.05 were considered significant. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001  (Student’s t test).

Chapter 2, in part, contains material being prepared for publication. Mack, Lauren; Leblanc, Mat; Zheng, Ye. “The role of Cryptochrome on regulatory T cell function”. The dissertation/thesis author was the primary investigator for this research.
Chapter 3: Cryptochromes are required for optimal peripheral homeostasis of regulatory T cells

3.1 Results

To determine the role of Cry on regulatory T cell development and homeostasis, we made CryDKO bone marrow chimeric mice. Upon analysis, these mice did not show a defect in the formation and expansion of lymphocyte compartment, including regulatory T cells (Data not shown). We further investigated the role of Cry on lymphocyte development and homeostatic fitness by transferring at 1:1 ratio of bone marrows isolated from Wildtype and CryDKO mice into irradiated Rag1 KO hosts and assessed B and T lymphocyte populations 6 weeks after reconstitution. We observed that the 1:1 ratio of Wildtype and CryDKO cell frequency was maintained in the B cell populations in the spleen (Figure 3.1a-b). In contrast, the frequencies of CryDKO CD8+ and CD4+ T cell populations were significantly reduced compared to their wildtype counterparts in the spleen and lymph node (Figure 3.1a-d). This competitive disadvantage of CryDKO T cells was more pronounced in the Treg compartment in which most Tregs were derived from the wildtype bone marrow (Figure 3.1a-d). By monitoring peripheral blood for lymphocyte frequency we confirmed that the deficiency of CryDKO T cells was constantly maintained over a four-month period after bone marrow reconstitution (Figure 3.2). This outcome could originate from
two possible sources; either a defect in thymic generation of Cry deficient T cells or a reduced capacity of mature CryDKO T cells to expand in the peripheral lymphoid organs. To determine if the reduced frequency of CryDKO T cells was in fact due to defective thymic development, we analyzed the frequency of Wildtype and CryDKO T cells from the thymus of the mixed bone marrow chimeras (Figure 3.1e-f). Thymic Wildtype and CryDKO T cells at all developmental stages were comparable, indicating that CryDKO T cells, in particular Tregs, have a defect in homeostatic expansion in the periphery. To directly investigate this premise, we transferred CD45.2 wildtype or CryDKO Tregs into immunodeficient recipient mice along with an equal number of CD45.1 wildtype Tregs and determined the ratio of transferred cells after 10 days of expansion (Figure 3.3a-c). The CD45.2 wildtype/CD45.1 wildtype ratio was maintained 10 days after adoptive transfer while the ratio of CD45.2 CryDKO/CD45.1 Wildtype had shifted dramatically. It was seen that there were about 3 times more Wildtype Tregs in the spleen and lymph nodes than CryDKO Tregs. These data indicate that CryDKO Tregs do in fact have a marked disadvantage in competitive peripheral expansion. Tregs can be specifically targeted for expansion by treatment with interleukin-2 (IL-2)/anti-IL-2 antibody complex due to the high expression of IL-2 receptor on Treg cell surface (Webster et al., 2009). To determine if IL-2 mediated expansion of CryDKO Tregs was also impaired, we injected IL-2/anti-IL-2 complex in Wildtype/CryDKO mixed bone marrow chimeric mice and determined the frequency of Wildtype and
CryDKO Tregs before and after expansion (Figure 3.3d). Wildtype Tregs expanded significantly more than the CryDKO Treg resulting in an increase in the frequency of wildtype Tregs and a proportional decrease in the frequency of CryDKO Tregs. This result demonstrated that IL-2 mediated peripheral expansion of Cry deficient Tregs is impaired. Taken together, these data revealed that Cry deficient T cells, particularly Tregs have an impaired capability for peripheral homeostatic proliferation in a competitive environment. It also suggests that the Cry deficient Tregs might be less functionally competent.

Chapter 3, in part, contains material being prepared for publication. Mack, Lauren; Leblanc, Mat; Zheng, Ye. “The role of Cryptochrome on regulatory T cell function”. The dissertation/thesis author was the primary investigator for this research.
3.2 Chapter Figures

Figure 3.1

Reduced Peripheral Treg development in the absence of Cryptochrome

1:1 Ratio of bone marrow (BM) from WT and CryDKO mice was transferred into Rag1KO irradiated hosts (a-f). Analysis of WT/CryDKO mixed bone marrow chimera mice in the spleen (a-b), lymph nodes (c-d), and thymus (e-f) 6 weeks after reconstitution. Cell frequencies within each donor-derived population (WT (Blue) and CryDKO (Red)) are shown by representative FACS plots (a,c,e) and graphed (b,d,f). n=4. Values represent the mean ± SD. (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001). Data are representative of three independent experiments.
Figure 3.2

Reduced Peripheral CryDKO Treg development in PBMC

1:1 Ratio of bone marrow (BM) from WT and CryDKO mice was transferred into Rag1KO irradiated hosts. Ratio of WT and CryDKO cell frequency of peripheral blood lymphocytes was plotted over time n=12. Data are representative of three independent experiments.
**Figure 3.3**

*Cryptochrome deficient Tregs have impaired peripheral expansion*

(a-c) Adoptive transfer of CD45.2 WT or CryDKO Treg cells into recipient mice compared to a CD45.1 WT Treg control was analyzed 10 days after transfer to determine relative rate of peripheral expansion. n=4. Representative FACS plots for WT (a) and CryDKO (b) Treg transfer in lymph nodes and spleen (a-b). Frequencies of CD45.2 WT or CryDKO (Blue) compared to CD45.1 Control Tregs (Yellow). (d) Relative increase in frequency of WT and CryDKO Tregs post-Treg expansion with IL-2/allL-2 antibody complex in WT/CryDKO mixed bone marrow chimeric mice. n=4. Values represent the mean ± SD. (***p ≤ 0.001). Data are representative of two independent experiments.
Chapter 4: Cry deficient Tregs have compromised suppressive capacity both *in vitro* and *in vivo*

4.1 Results

Since it was observed that the development of Tregs was the most affected out of the lymphocyte population examined, we wanted to assess the suppressive capacity of Cry deficient Tregs. We evaluated the capability of CryDKO Tregs to suppress responder T cell proliferation *in vitro* (Figure 4.1). Cry deficient Tregs are notably less capable of suppression of T cell proliferation in a dose dependent manner. This is not caused by a difference in Treg cell survival, at the end of assay equal numbers of wildtype and CryDKO cells were seen (Data not shown). This suggests that not only is there a deficiency in CryDKO Tregs in peripheral expansion but that they are also functionally impaired. To assess the role of Cry in Treg suppression further we quantified the functionality of Tregs that were ectopically expressing Cry1 *in vitro* (Figure 4.1). It was seen that Cry1 overexpressing Tregs were more suppressive than their control counterparts in a dose dependent manner. These data contraindicated that Cry is necessary for optimal Treg function *in vitro*.

We next tested if these findings can be extrapolated *in vivo*. Mixed bone marrow chimera mice were generated by transferring bone marrow from Foxp3 deficient mice mixed with either wildtype or CryDKO bone marrow (Figure 4.3). 

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Since Foxp3 is the determining transcription factor for Treg generation, no Tregs would develop from this Foxp3-deficient bone marrow cells while development of all other hematopoetic lineages are unaffected (Fontenot et al., 2003). This allowed us to assess the affect of Cry deficiency specifically on the Treg population without affecting any other cell type. CryDKO:Foxp3KO mice developed symptoms of immunopathology as early as five weeks after reconstitution. Analysis of the lymphoid organs showed that the mice reconstituted with CryDKO:Foxp3KO bone marrow had increased activation of CD4+ and CD8+ T cells in both the lymph nodes and spleen (Figure 4.3a-d). Cytokine staining revealed that Interferon gamma (IFN-g) production by CD4+ T cells was markedly increased (Figure 4.3e-f). This data was not due to the fact that that the development of Tregs in these mice were impaired as there were equal frequencies of Foxp3+ CD4 T cells in CryDKO:Foxp3KO chimeric mice compared to WT:Foxp3KO controls (Figure 4.3g-h). Due to increased immune cell activation in the CryDKO:Foxp3KO chimeric mice, they develop immunopathology that is seen in peripheral organs, most notably in the stomach, liver, heart, and skin; measured by immune cell infiltration and inflammation (Figure 4.4). This indicates that Cry deficient Tregs are defective at suppressing T cell activation and maintaining homeostasis in vivo, which leads to immunopathology. These data demonstrate the Cry deficient Tregs are less suppressive both in vitro and in vivo.
We wanted to ensure that the role of cry in Treg function \textit{in vivo} was cell-intrinsic. Therefore, we used a model of T cell induced colitis in which the induction of colitis can be mediated by Treg suppression of T cell activation and proliferation. When only responder T cells are transferred to recipient mice these mice rapidly lose weight until becoming moribund (Figure 4.5a). However, when Tregs are co-transferred along with responder T cells colitis induction is prevented and recipient mice maintain their weight over time. When Cry deficient Tregs were transferred instead it was seen that these mice are not as capable at maintaining their weight especially at late timepoints of the experiment indicating that Cry deficient Tregs are less capable at suppression \textit{in vivo}. To ensure that this was not caused by a difference in Treg frequency in recipient mice, Treg frequency was assessed in the gut draining lymph node (mesenteric lymph node), lymph nodes, and spleen at the end of the assay (Figure 4.5b). Comparable frequencies of Tregs were seen in all tissues examined indicating that the difference in weight was in fact due to differences in Treg suppression.

We also evaluated the absolute numbers of transferred responder T cells that proliferate two weeks after transfer to assess Treg suppression (Figure 4.6a). In this assay we transferred responder T cells alone or with Wildtype or CryDKO Tregs into lymphopenic recipient mice and assessed the absolute numbers of transferred responder cells after two weeks post transfer. When mice only received responder T cells, the absolute number of transferred cells increased because they were not being inhibited by Tregs. The proliferation of
responder T cells was decreased when co-transferred with Wildtype Tregs. However, when Treg were deficient in Cry the inhibition on responder T cell proliferation was decreased which was most evident in mesenteric lymph node, which is where many of these cells migrate after transfer. These data indicate that Cry deficient Tregs have a compromised suppressive capacity and that this is a cell-intrinsic defect.

Chapter 4, in part, contains material being prepared for publication. Mack, Lauren; Leblanc, Mat; Zheng, Ye. “The role of Cryptochrome on regulatory T cell function”. The dissertation/thesis author was the primary investigator for this research.
4.2 Chapter Figures

**Figure 4.1**

**Cryptochrome deficient Tregs have less capacity to suppress T cell proliferation in vitro**

Suppression of proliferation of CFSE labeled WT naïve (CD44loCD62Lhi) T effector (Teff) cells by WT and CryDKO Tregs, represented as dilution of CFSE in Teff cells cultured with Tregs at indicated ratio. Dividing Teff cells are those that have undergone at least one cell division indicated by dilution of CFSE. Values represent the mean ± SD. (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001). Data are representative of four independent experiments.
Figure 4.2

Ectopic expression of Cryptochrome in Tregs confer increased capacity to suppress T cell proliferation \textit{in vitro}

Suppression of proliferation of CFSE labeled naïve (CD44loCD62Lhi) T effector (Teff) cells by Tregs overexpressing Cry1 or a control vector. Represented as dilution of CFSE in Teff cells cultured with Tregs at indicated ratio. Dividing Teff cells are those that have undergone at least one cell division indicated by dilution of CFSE. Values represent the mean ± SD. (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001). Data are representative of four independent experiments.
Figure 4.3

Loss of Cryptochrome in Tregs leads to T cell activation and cytokine production in vivo

1:1 Ratio of WT or CRYDKO BM with Foxp3KO BM was transferred into Rag1KO irradiated hosts (a-i) Analysis of WT/Foxp3KO (Blue) and CryDKO/Foxp3KO (Red) mixed bone marrow chimeric mice 6 weeks after reconstitution. Frequency of activated (CD44hiCD62lo) CD4 T cells (a-b), activated CD8 T cells (c-d), Interferon gamma production in CD4 T cells (e-f) and Treg (g-h) was determined in lymph nodes and spleen. n=6-7. Values represent the mean ± SD. (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001). Data are representative of three independent experiments.
Loss of Cryptochrome in Tregs leads to immunopathology

1:1 Ratio of WT or CRYDKO BM with Foxp3KO BM was transferred into Rag1KO irradiated hosts. Analysis of WT/Foxp3KO (Blue) and CryDKO/Foxp3KO (Red) mixed bone marrow chimeric mice 6 weeks after reconstitution. Representative H&E staining of mixed bone marrow chimeric mice of stomach, liver, heart, and skin. Scale Bar represents 100μm in stomach, heart, and skin. Scale bar represents 500μm in liver. n=6-7. Data are representative of three independent experiments.
Figure 4.5

**Cryptochrome deficient Tregs have less capacity to suppress T cell induced colitis in vivo.**

Naïve T responder cells were transferred to Rag1KO mice alone or in combination with WT or CryDKO Tregs. Changes in body weight of Rag1KO recipients over time are shown (a). Frequency of Tregs in lymph nodes, mesenteric lymph node, and spleen (b). n=5-6. Data are representative of 3 independent experiments. Data are representative of 3 independent experiments. Values represent the mean ± SD. (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001).
Figure 4.6

Cryptochrome deficient Tregs have less capacity to suppress T cell proliferation in vivo.

Naïve T responder cells were transferred to Rag1KO mice alone or in combination with WT or CryDKO Tregs. Spleen, lymph nodes was analyzed by FACs analysis 14 days later for absolute number of transferred T responder cells (a) and frequency of Tregs (b). n=5. Data are representative of 3 independent experiments. Values represent the mean ± SD. (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001).
Chapter 5: Molecule Mechanism of Cry in Tregs

5.1 Results: Role of Cry in cAMP production

It has been reported that cells that lack expression of Cry have upregulation of cyclic adenosine monophosphate (cAMP) production (Narasimamurthy et al., 2012; E. E. Zhang et al., 2010). Since cAMP production has previously been linked to Treg function, the relative levels of cAMP in CryDKO Tregs was determined within in vitro activated Tregs (Figure 5.1a). There was measurably more intracellular cAMP in CryDKO Tregs compared to wildtype control Tregs, which replicates what has been reported in other cell types. The previously reported studies have indicated that cytoplasmic Cry is able to inhibit production of cAMP directly by binding to proteins involved in G-protein coupled receptor (GPCR) signaling. However, the binding partner of Cry in yet to be conclusively determined due to the fact that both Adenylyl cyclase and the Gs alpha subunit (Gsα) have been indicated. Specific activators of GPCR signaling were used to determine where in the signaling pathway is inhibited by Cry; Forskolin activates adenylyl cyclase while prostaglandin E2 (PGE2) activates the GPCR. The cAMP increase seen in Cry deficient Tregs is unaltered with Forskolin treatment but in eliminated with treatment with PGE2, which indicates that Cry is targeting the GPCR pathway upstream of the adenylyl cyclase. The role of increased cAMP levels on Treg function as yet to be
elucidated; to investigate its role we utilized two methods to artificially increase intracellular cAMP levels in Tregs and measured the in vitro suppressive capacity. First, Intracellular cAMP levels of Tregs was increased by ectopic expression of $G_s\alpha$ subunit gene (GNAS). The capability of $G_s\alpha$ overexpression had no effect of the capability of these Tregs to suppress responder T cell proliferation in vitro (Figure 5.1b). We also utilized the cAMP antagonist (3′,5′-hydrogen phosphorothioate adenosine triethylammonium, RP-cAMPS) to determine if cAMP overproduction is responsible for the decreased suppression seen in Cry deficient Tregs (Figure 5.1C). No affect was seen on Treg Suppression in either CryDKO or Wildtype Tregs which demonstrates that cAMP overproduction is most likely not responsible for Cry decreased suppressive capacity.

5.2 Results: Role of Cry in regulation of mitochondria and oxidative phosphorylation

We next investigated the gene expression changes that were present in CryDKO Tregs that might be related to their defective suppression function. We performed RNA sequencing of Wildtype and CryDKO Tregs and used cluster analysis to identify the biological processes that were the most significantly altered in CryDKO Tregs (Figure 6.1a). The two major gene clusters that were altered in CryDKO Tregs were processes involved in rhythmic processes and mitochondrial respiration. Both of these groups had increased expression in CryDKO Tregs compared to Wildtype (Figure 6.1b). Since Cry is a negative
regulator of the circadian molecular clock it was expected that in its absence upregulation of Clock related genes would occur. Previous studies have identified that although T cells do maintain a cell-intrinsic oscillation of circadian clock genes disruption of these genes does not affect T cell function (Hemmers and Rudensky, 2015). However the affect of increased mitochondrial electron transport on Treg function has yet to be investigated. Our data agree with previous findings that Cry deficient mouse embryonic fibroblasts have increased mitochondrial respiration measured by fatty acid oxidation and levels of NAD+ (Peek et al., 2013). A number of the genes that have increased expression in this cluster are directly involved in mitochondrial electron transport and might alter mitochondrial energy production in Cry deficient Tregs. It was thus important to determine if these gene expression changes manifest into measurable differences in mitochondrial capacity in Tregs.

To measure the effect of the upregulation of genes involved in mitochondrial electron transport in CryDKO Tregs, we tested if it resulted in changes in cellular energy production. Energy can be produced through one of the two major pathways, oxygen dependent (mitochondrial respiration) and oxygen independent (glycolysis). These two energy production pathways were quantified in wildtype and CryDKO Tregs using a Seahorse XF analyzer. Mitochondrial respiration was determined by measuring the oxygen consumption rate over time. Our results showed that both the basal respiratory capacity and the spare respiratory capacity (measured after the addition of FCCP) of CryDKO
Tregs was significantly higher compared to the wildtype control (Figure 6.2a). In contrast, the levels of glycolysis in CryDKO and Wildtype Tregs were comparable (Figure 6.2b).

Increased mitochondrial respiratory capacity could be caused by either increased numbers of mitochondrion or increased inner mitochondrial membrane potential or both. To distinguish these possibilities, we determined the mitochondrial content of Wildtype and CryDKO Tregs by measuring the ratio of mitochondrial DNA (mtDNA) to genomic DNA (gDNA) (Figure 6.2c). There was no significant difference in the mitochondrial content between CryDKO and Wildtype Tregs. Next, we measured mitochondrial membrane potential of Wildtype and CryDKO Tregs by using a membrane potential sensitive fluorescent dye DiOC$_2$. Interestingly, CryDKO Tregs have significantly higher mitochondrial membrane potential compared to Wildtype Tregs (Figure 6.2d-e). This indicates that, in the CryDKO Tregs, the increase in the expression of genes involved in electron transport across the mitochondrial inner membrane leads to increased mitochondrial respiration due to elevated mitochondrial membrane potential.

Increased mitochondrial respiration can promote production of reactive oxygen species (ROS) as a byproduct of the electron transport chain. It has been reported that increased ROS levels in T cells and many other cell types can lead to decreased biological function (Belikov et al., 2015). Thus we measured the levels of ROS in Cry deficient Tregs (Figure 6.2f-g). Interestingly, instead of increasing, ROS production in CryDKO Tregs showed a slight decrease. This
could be explained by increased expression of superoxide dismutase 2 (Sod2) in CryDKO Tregs. Sod2 is an enzyme that is able to transform superoxide to O$_2$ and ameliorate the buildup of ROS in cells (Figure 6.1b). Therefore, the dysfunction of Cry deficient Tregs cells is not caused by increased ROS production.

Here we have demonstrated that CryDKO Tregs have increased mitochondrial respiratory capacity, which may affect Treg function. Although we have not demonstrated a direct link between these two properties, the most prominent gene expression alterations in CryDKO Tregs were changes in circadian associated genes and those involved in mitochondrial respiration, which are most likely responsible for the dysfunction of Cry deficient Tregs.

Chapter 5, in part, contains material being prepared for publication. Mack, Lauren; Leblanc, Mat; Zheng, Ye. “The role of Cryptochrome on regulatory T cell function”. The dissertation/thesis author was the primary investigator for this research.
5.3 Chapter Figures

(a) Metascape cluster enrichment analysis for GO biological processes genes differentially expressed in WT and CryDKO Tregs
(b) Heat map of 2 gene clusters of differentially expressed genes in WT and CryDKO Tregs. Relative fold change was normalized to the average of each row in the matrix.

Figure 5.2

Cryptochrome deficient Tregs have increased expression of genes involved in mitochondrial respiration

(a) Metascape cluster enrichment analysis for GO biological processes genes differentially expressed in WT and CryDKO Tregs (b) Heat map of 2 gene clusters of differentially expressed genes in WT and CryDKO Tregs. Relative fold change was normalized to the average of each row in the matrix.
Figure 5.3

Cryptochrome deficient Tregs have increased mitochondrial respiration caused by increased mitochondrial membrane potential.

OCR of in vitro activated WT and CryDKO Tregs was measured under basal conditions and in response to oligomycin (Oligo), FCCP, and Antimycin A + Rotenone. (b) ECAR of in vitro activated WT and CryDKO Tregs was measured under basal conditions. n=5. Data is representative of 4 independent experiments. (c) Mitochondrial content was measured by the ratio of mtDNA: gDNA of in vitro activated WT and CryDKO Tregs. (d-e) Mitochondrial membrane potential was measured of in vitro activated WT and CryDKO Tregs by FACs staining with DiOC₂ with or without the mitochondrial uncoupler, CCCP. (f-g) Reactive oxygen species production by in vitro activated WT and CryDKO Tregs was measured by FACs staining with CM-H₂CFDA. Data is representative of 3 independent experiments. n=3. Values represent the mean ± SD. (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001)
Figure 5.1

Increased cAMP in Cry deficient Tregs but does not affect Treg function.

(a) cAMP concentration of *in vitro* activated WT and CryDKO Tregs in untreated cella or after the addition of Forskolin or PGE2. n=3. Data are representative of four independent experiments. (b) Suppression of proliferation of CFSE labeled naïve (CD44loCD62Lhi) T effector (Teff) cells by Tregs overexpressing GNAS or a control vector. Represented as dilution of CFSE in Teff cells cultured with Tregs at indicated ratio. Dividing Teff cells are those that have undergone at least one cell division indicated by dilution of CFSE. n=3. Data are representative of two independent experiments. (c) Suppression of proliferation of CFSE labeled naïve (CD44loCD62Lhi) T effector (Teff) cells by Tregs alone or with the addition of the cAMP antagonist BR-cAMPS. n=3. Data are representative of two independent experiments. Values represent the mean ± SD. (*p ≤ 0.05, **p ≤ 0.01)
Chapter 6: Discussion

6.1 Summary of findings

My thesis research has focused on understanding the role of Cry, the main circadian transcriptional repressor, in Treg cell development and function. This was done in three parts: first, I investigated the affect of Cry deficiency on Treg development and proliferation \textit{in vivo}; second, I evaluated the role of Cry in Treg function; finally, I analyzed potential mechanism by which Cry is important in Treg.

This project identifies Cry as a novel gene important for Treg development and function while further projects are needed to confirm the mechanism by which Cry is necessary in Treg.

To establish the role of Cry in Treg development we used a model of competitive homeostatic proliferation in which both Wildtype and CryDKO cells rapidly proliferated in mixed bone marrow chimera mice. In this model we saw that CryDKO T cells were present at a lower frequency in all T cell compartments analyzed; CD8, CD4, and Treg. However, the strongest defect was seen in the Treg compartment where the vast majority of the Tregs came from wildtype cells. This was not due to a difference in Treg development in the thymus because an equal ratio of Wildtype and CryDKO T cells were seen at ever stage of T cell development; double negative, double positive, single positive, and in the Treg compartment. These results could have been obtained for two possible reasons: CryDKO Tregs undergo higher rates of cell death in the periphery or CryDKO
Tregs have a lower proliferative capacity than their wildtype counterparts. Experiments done to test the rate of apoptosis has given no identification that CryDKO Tregs have increased cell death. Both Wildtype and CryDKO Tregs grow at equal rates in ex vitro culture and all long-term Treg transfer experiments clearly demonstrate that CryDKO Tregs are able to establish equal frequencies of Tregs in vivo when not in a competitive environment. Thus it is most likely that CryDKO Tregs are less proliferative compared to their Wildtype counterparts. We tested this directly in two experimental models: one; of competitive homeostatic expansion; second, using IL-2 mediated expansion of Tregs. It was seen in both experiments that the expanded Tregs frequency were skewed towards wildtype Tregs, demonstrated that wildtype Tregs are more proliferative in vivo than CryDKO Tregs. Since preserving Treg homeostasis is essential for maintenance for the immune system, Cry deficient cells may be less capable of mounting an efficient immunosuppressive response when rapid expansion of Tregs is needed.

It was important to establish if CryDKO Tregs maintained their suppressive capacity. We measured the in vitro capacity of CryDKO Tregs to suppress effector T cell proliferation, which showed that they had significantly less suppressive capacity than wildtype Tregs. The converse was seen when Tregs had ectopic expression of Cry1, these Tregs were more suppressive then control Tregs. Next, we tested the in vivo suppressive capacity of CryDKO Tregs in vivo by making mixed bone marrow chimera mice where only the Treg compartment was completely deficient in Cry expression. It was seen that when
Tregs lacked Cry expression in vivo mice develop immunopathology characterized by increased activation of T cells and more cytokine production by these cells. This was not due to the fact that mice with Cry deficient Tregs had fewer Tregs since the frequency of Tregs was unaltered. To ensure that these results were a result of a Treg intrinsic defect we used a used a model of in vivo Treg suppression, which utilized transferred Tregs to suppress the development of T cell induced colitis. CryDKO Tregs were less capable of suppressing development of colitis in host mice measured by weight loss over the course of the experiment. This data was replicated when immunosuppression capacity of CryDKO Tregs was directly measured in vivo by assessing the total numbers of T cells that proliferated after co-transfer with wildtype or CryDKO Tregs. These data demonstrated that CryDKO Tregs are less capable of suppressing the immune system both in vitro and in vivo.

Tregs deficient in Cry develop at lower frequencies in a competitive environment and are less suppressive. To uncover the mechanism that could be responsible for these defects we analyzed the RNA expression changes in CryDKO Tregs. The major pathways that were affected by the lack of Cry expression were genes involved in mitochondrial respiration and those in the circadian clock. It has been previously reported that mice deficient in a cell intrinsic circadian clock had no changes on T cell development or function (Hemmers and Rudensky, 2015). Cry has been shown to affect many cellular pathways outside of the circadian clock including cellular metabolic pathways
(Koike et al., 2012; Peek et al., 2013). Therefore we decided to analyze the changes seen in mitochondrial energetic pathways, where the many genes directly involved in the electron transport chain were significantly increased in CryDKO Tregs. We observed that CryDKO Tregs have higher levels of basal mitochondrial respiration as well as spare respiratory capacity while the rate of glycolysis was unchanged. This was not due to an increase in mitochondrial mass but to higher mitochondrial membrane potential. Increased mitochondrial membrane potential is associated with increased production of Reactive Oxygen Species (ROS), which as been associated with has been implicated in T cell dysfunction (Belikov et al., 2015). Therefore, we decided to test if the production of ROS in CryDKO Tregs was increased and could be responsible for the Treg dysfunction seen. However no changes in production of Hydrogen Peroxide was seen compared to Wildtype Tregs. This could be due to the fact that CryDKO Tregs may have a compensatory mechanism; upregulation of Superoxide Dismutase 2 was seen in CryDKO Tregs. The increase of SOD2 would be able to convert any superoxide products from increased mitochondrial activity into inert oxygen molecules. Therefore it is unlikely that ROS production is responsible for the defect seen in CryDKO Tregs. There have been previous studies that have looked at the affect cellular metabolism in Tregs but none have identified a phenotype of increased mitochondrial respiration.

Although no changes in CREB related expression patterns was seen in RNA expression analysis it has been reported previously that Cry deficient cells
have increased cAMP (Narasimamurthy et al., 2012; E. E. Zhang et al., 2010). Therefore we analyzed the levels of cAMP expression in CryDKO Tregs and we saw that there was in fact a significant increase in cAMP production in these cells. However when cAMP was artificially altered, increased by ectopic expression of GNAS or inhibited by addition of a cAMP antagonist no measurable affect was seen on Treg suppression. Therefore, we concluded that most likely the increase in cAMP seen in CryDKO Tregs does not play an important role in their suppressive capacity.

6.2 Circadian Clock in T cells and Tregs

Diurnal variations in T cell responses and circulating T cells was first discovered many years ago. However, it is unknown if this is due to the control of the central circadian clock emanating for the SCN through hormones and other mechanisms of long range signaling or due to the importance of a T cell intrinsic molecular clock. It has been shown that T cell do in fact have oscillation of the genes involved in control of the circadian clock but their importance as yet to be revealed (Bollinger et al., 2011). One study has suggested that eliminating RE-VERB from T cells decreases their ability to differentiate into TH17 cells (Yu et al., 2013). This study also showed that in a model of circadian disruption resulting from chronic jetlag/time shifting an increase in TH17 cells was observed in the gut. In contrast, another study showed that a T cell deletion of BMAL1 had no affect on the T cell response to bacterial or viral infection (Hemmers and Rudensky, 2015). In a competitive environment BMAL1 deficient T cells
produced slightly less IL-2 production than their wildtype counterparts. However, there was still variation in the T cell IL-2 response at different times of the day which shows that the cytokine response in T cells is controlled by a cell extrinsic factor. Therefore, there is still much to be discovered about the interplay between the circadian clock and T cell function.

It is important to note that only Cry and BMAL1 have been shown to completely and immediately disrupt the circadian clock showing their important role in the maintenance of the circadian clock but also highlights the complex interactions in this biological system. The genes involved in the circadian molecular clock form a complex interlocking loop of regulation that includes transcriptional and post-translational regulation of molecules within the core clock as well as many biological processes outside of the clock. Each of components of the molecular clock, particularly Cry and RE-VERB are promiscuous as they affect of many molecular pathways within the cell. Cry has been found to bind to thousands of sites outside of the Core-Clock related genes (Koike et al., 2012). This highlights the fact that each component of the molecular circadian clock holds a unique role within the circadian clock but also on its affect on genes outside of the clock. Therefore it is possible that the reason why a discrepancy is seen in the studies of the circadian clock on T cell function, this could be due to specific impact of the particular gene disrupted.

To date this is the first time that the affect of the cell intrinsic circadian clock as been studied in Tregs. Previously human Tregs have been analyzed for
their suppressive capacity for daily variation and it was discovered that there was a diurnal variation in their ability to suppress T cell proliferation (Bollinger et al., 2009). However, this study does not address if this variation is due to the central clock or due to the Treg intrinsic clock. We addressed this question by disrupting the circadian clock specifically in Tregs, which lead to a decrease in Treg development and function. In experiments not shown here, we have analyzed the affect of BMAL1 deficiency in Tregs and found no affect either in vitro or in vivo. This suggests that the defect in CryDKO Treg development and function is not directly due to the disruption of transcriptional control of the core clock related genes but due to the output of the circadian clock on other biological processes. Since the interplay of circadian clock and other affected biological pathways is intermingled so tightly it is hard to discriminate the direct and indirect affects of the circadian clock on Treg homeostasis.

6.3 Cellular Metabolism in Tregs

As stated previously, the cell intrinsic circadian clock affects many biological processes. Studies have indicated that at least 10% of all transcripts are regulated in a circadian manner in tissues and this percentage increases to close to 20% of genes involved in metabolic processes (Eckel-Mahan et al., 2012; Minami et al., 2009; Storch et al., 2002). Along with alteration in the expression of genes involved in the circadian clock we also observed deregulation of genes involved in mitochondrial respiration. Interestingly, the role
of cellular metabolism on the development and function of immune cells has become a field with rising interest.

Much research on Tregs has been done utilizing *in vitro* differentiated Tregs, these iTregs have been generated from naïve T cells in vitro using the addition of TGF-β and are more similar to pTregs which make up only 10-30% of the Tregs in vivo and are only generated in specific mucosal interfaces with the environment (Thornton et al., 2010; Weiss et al., 2012; Yadav et al., 2012). Therefore it is important to study Tregs isolated *ex vivo* to ensure the proper representation of Tregs. Studies that have looked specifically at cellular metabolism of Tregs *in vivo* have identified a much more complex network of metabolic control than what was first identified in iTreg populations. Initially studies in iTregs indicated they use Fatty Acid Oxidation (FAO) and are not affected by inhibition of glycolytic pathways while other differentiated T cells use primarily glycolysis (Macintyre et al., 2014; Michalek et al., 2011; Pollizzi and Powell, 2014; Shi et al., 2011). Some studies however have suggested that Tregs derived *in vitro* may also need glycolysis (De Rosa et al., 2015).

However, when the cellular metabolism of Treg were assessed *in vivo*, inhibition of FAO had no affect of Treg (Byersdorfer et al., 2013). Also, proteomic analysis suggests that *Ex vivo* activated Tregs rely on both glycolysis and FAO while conventional T cells primarily use glucose metabolism (Procaccini et al., 2016). This indicates that the metabolic phenotype of iTregs and tTregs may diverge. Similarly, while mTOR inhibition generates more iTregs in vitro it is also
important for the maintenance of Treg stability in vivo (Zeng et al., 2013). As mTOR signaling increases glycolysis as well as promotes cholesterol and lipid metabolism, it suggests that glycolysis may be important for Treg metabolism in vivo. However, aberrant mTOR signaling by inhibiting autophagy also results in Treg apoptosis and development of immunopathology (Wei et al., 2016). These studies suggest that metabolic pathways in Tregs need to be highly regulated for maintenance of Treg stability and proliferation in vivo. These studies have investigated increases/decreases in glycolysis and decreases in FAO; as of yet there have not been any studies that address the affect of increased OXPHOS in Tregs. As it seems that Tregs rely on both glycolysis and OXPHOS it is reasonable to assume that aberrant upregulation of OXPHOS in CryDKO Tregs could be a reason for their decreased immunosuppressive abilities. Further studies in our laboratory will attempt to further investigate the role of OXPHOS in Treg function.

6.4 Conclusion

In this study we investigated the role of Cry in Treg development and function. It was observed that Tregs lacking Cry are not as proliferative or immunosuppressive as their wildtype counterparts. This highlights a novel role of a circadian clock gene in T cell function and homeostasis. Further investigations are need to determine if the defects seen are caused by the direct disruption of the Treg intrinsic circadian clock, Cry’s affect outside of the clock onto Treg cellular metabolism, or the interplay of the two. All together, this project has
identified a novel component necessary for Treg cell homeostasis and reveals that there are many more complexities waiting to be discovered in Treg biology.
REFERENCES


Hirano, A., Yumimoto, K., Tsunematsu, R., Matsumoto, M., Oyama, M., Kozukahata, H., Nakagawa, T., Lanjakornsiripan, D., Nakayama, K.I., Fukada, Y., 2013. FBXL21 Regulates Oscillation of the Circadian Clock through


Immunity 41, 75–88.


Prokopenko, I., Langenberg, C., Florez, J.C., Saxena, R., Soranzo, N., Thorleifsson, G., Loos, R.J.F., Manning, A.K., Jackson, A.U., Aulchenko, Y.,


1029–1033.


