UNIVERSITY OF CALIFORNIA SAN DIEGO

Regulators of a regulator: microRNAs in control of regulatory T cell biology

A Dissertation submitted in partial satisfaction of the requirements
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Biology

by

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The Dissertation of Jiayi Dong is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

University of California San Diego

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DEDICATION

To Mom and Dad, for everything you've given me

EPIGRAPH

Three passions, simple but overwhelmingly strong, have governed my life:
the longing for love,
the search for knowledge,
and unbearable pity for the suffering of mankind.

— Bertrand Russell, *What I Have Lived For*

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ABSTRACT OF THE DISSERTATION

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MicroRNAs (miRNAs), a class of small non-coding RNA molecules, are key nodes in regulatory networks that fine-tune gene expression and orchestrate diverse signaling pathways that shape immune responses. Since the first study of Dicer-dependent miRNA pathway in regulatory T (Treg) cells in more than a decade ago, miRNAs have been shown to serve as key

regulatory elements in controlling the development and function of this specialized $CD4^+$ T cell subset that is indispensable for maintaining immune tolerance and homeostasis. Nevertheless, despite the rapidly accumulating knowledge of the role of miRNAs in Treg cells, our understanding of the intricate regulations of miRNAs and their targets in regulating different aspects of Treg cell biology remains limited.

In this work, we demonstrate a critical role for miR-155, which ensures proper Treg cell development in the thymus. Specifically, in addition to the previously reported function of miR-155 in conferring Treg cell competitive fitness, we uncover a novel miR-155-TGFβ axis in the thymic medulla that determines medullary thymic epithelial cell (mTEC) maturity and, consequently, the quantity of thymic Treg cells. In addition to miR-155, we also identify another miRNA family, miR-15/16 that plays an important role in restricting Treg cell function and homeostasis. To this end, we find that the miR-15/16 family, miR-15b/16-2 in particular, is specifically up-regulated in Treg cells in the thymus. Nevertheless, this miRNA family does not seem to play a significant role in regulating thymic Treg cell development. Rather, it is important to control effector Treg (eTreg) cell differentiation and function in the periphery. Mechanistically, the miR-15/16 family targets a network of genes, including a transcription factor, IRF4 that are critical for the establishment of the eTreg cell program.

Collectively, our findings highlight both cell-extrinsic and –intrinsic roles of miRNAs mediated by miR-155 and miR-15/16, respectively in controlling Treg cell biology. Our work not only reveals novel mechanisms by which miRNAs regulate Treg cell-mediated immune tolerance but also provides molecular insights that will undoubtedly aid in developing innovative strategies targeting Treg cells to treat a wide range of immunological disorders.

CHAPTER I: INTRODUCTION

Our immune system needs to maintain a delicate balance to generate an effective response against pathogens and cancerous cells, without mounting unwanted immune activation that leads to deleterious inflammation and tissue damage. One of the key immune cell populations involved in maintaining this immune balance is regulatory T (Treg) cell, a dedicated T cell subset crucial for the negative regulation of the immune response. To date, many different transcriptional and epigenetic mechanisms have been revealed to regulate the development and function of Treg cells. Among them, microRNAs (miRNAs), a class of small non-coding RNA molecules, have been intensively investigated for their roles in controlling gene expression at both post-transcriptional and translational levels in the immune system.

In this chapter, I will focus on the regulatory mechanisms that govern Treg cell development and function, with a strong emphasis on the roles of miRNAs in modulating these processes.

1.1 Regulatory T Cell Biology

Treg cells are a distinct $CD4^+$ T lymphocyte population that is crucial for sustaining self-tolerance and immunological homeostasis. The master transcription factor Foxp3, a protein encoded on the X-chromosome, is a lineage factor that defines Treg cell identity and ensures the maintenance of their immunosuppressive function (Sakaguchi et al., 2008). Foxp3 mutations in humans are closely associated with severe autoimmune disorders, known as immunodysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) (Bennett et al., 2001). Similarly, Foxp3 deficiency leads to systemic inflammation and fatal multi-organ autoimmunity in mice, usually by 3 weeks of age (Kim et al., 2007). Thus, the proper development and function of Treg

cells are indispensable for the maintenance of immune tolerance and homeostasis, and aberrant Treg cell numbers or suppressive functions have been linked to a variety of immune disorders, such as type 1 diabetes, rheumatoid arthritis, inflammatory bowel disease, systemic lupus erythematosus, and multiple sclerosis (Dejaco et al., 2006).

The majority of Treg cells are naturally generated in the thymus (referred to as tTreg cells or nTreg cells). However, conventional $CD4^+$ T (Tconv) cells found in the periphery can also convert into Foxp3-expressing T cells and acquire Treg cell functions, both in vitro, known as "induced Treg" (iTreg) cells and in vivo, known as "peripherally induced Treg" (pTreg) cells (Elkord, 2014; Luo & Li, 2013).

The generation of tTreg cells relies on stepwise differentiation and maturation within the thymic microenvironment, which can be separated into cortex and medulla. During thymic development, immature $CD4^+CD8^+$ double-positive (DP) thymocytes go through positive selection for functional TCRs that recognize self-peptide: MHC class II presented on cortical thymic epithelial cells (cTEC) and become CD4 single-positive (SP) thymocytes. The resulting CD4SP thymocytes are subsequently subjected to negative selection against autoreactivity in the thymic medulla, where T cells with low affinity to self-antigens will pass the selection and become Tconv cells, while those with strong affinity will be eliminated via apoptosis. During this process, certain CD4SP thymocytes acquire Treg cell fate and Foxp3 expression, thereby circumventing autoreactivity. Thereafter, they travel to peripheral lymphoid organs as $CD4^+Foxp3^+$ Treg cells (Hsieh et al., 2012).

Thymic Treg cell development is driven by TCR stimulation coupled to the CD28 co-stimulatory signal (Salomon et al., 2000), as well as γc cytokine signals that promote their survival and progression into a mature Treg stage (Lio & Hsieh, 2008; Santamaria et al., 2021). A prevailing

tTreg development model implies a two-step process. Strong TCR signals trigger the expression of CD25 in CD4SP thymocytes, resulting in their development into $CD25^{+}Foxp3^{-}$ Treg precursors (step 1). The expression of CD25 (also known as IL2ra) makes Treg precursor cells receptive to γc cytokines, particularly IL-2, which rescue them from apoptosis, leading to their Foxp3 induction and further development into mature Treg cells (step 2) (Lio & Hsieh, 2008). Complementarily, a recently identified $Foxp3^{lo}$ Treg precursor population develops upon weaker TCR signals, and likely relies on IL-15 and IL-2 for their survival and differentiation into mature Treg cells (Owen et al., 2019).

As aforementioned, Treg cell survival and development in the thymus largely depends on a combination of cell-extrinsic signals provided by the thymic microenvironment as well as residing cells. For example, dendritic cells and self-reactive CD4SP thymocytes produce IL-2 (Hemmers et al., 2019; Weist et al., 2015), and thymic stromal cells, mainly TECs including cTECs and mTECs, produce IL-15 (Cui et al., 2014), two γc cytokines critical for Treg cell survival and development. In addition, the production of TGF-β by cTECs and apoptotic thymocytes has been implicated in the early development of tTreg cells at postnatal days 3-5 (Chen et al., 2001; Liu et al., 2008; Takahama et al., 1994). In the thymic medulla, mTECs promote tTreg cell generation and shape their TCR repertoire through their expression of co-stimulatory molecules CD80/CD86, MHC class II and autoimmune regulator (Aire), with the latter being essential for the expression of tissue-restricted-antigens (TRA) to guide negative selection and thymic Treg cell development (Abramson & Anderson, 2017; Anderson et al., 2005; Aschenbrenner et al., 2007).

After maturation, tTreg cells exit the thymus as naive Treg cells, which are defined by the expression of CD62L or CCR7 that enables their homing to the secondary lymphoid organs (Smigiel et al., 2014). In response to antigens and a variety of environmental cues in the periphery,

Treg cells can undergo further differentiation to acquire distinct phenotypes and effector molecules, which lead to the formation of different Treg cell subsets. In this regard, Treg cells are highly heterogeneous, depending on their differentiation stages, tissue residency, and their expression of functional molecules.

One example of Treg cell heterogeneity can be illustrated by a classic division of central and effector Treg cells, based on their distinct characteristics. Central Treg (cTreg) cells (defined as $CD44^{lo}CD62L^{hi}$) express high levels of the chemokine receptor CCR7, which enable them to actively recirculate through lymphoid organs. These cells express higher levels of CD25 and are thought to rely on IL-2 signaling for their maintenance. On the other hand, effector Treg (eTreg) cells (defined as $CD44^{hi}CD62L^{lo}$) depend on continued signaling through the co-stimulatory receptor ICOS signaling for their maintenance at non-lymphoid tissues and correspondingly express high levels of ICOS. In addition, these cells have highly proliferative capacities with marked expression of Ki67. However, they also express low levels of anti-apoptotic molecule Bcl-2, which serves as a compensatory mechanism that prevents the overproduction of effector Treg cells (Smigiel et al., 2014). eTreg cells are thought to differentiate from cTreg cells and exhibit enhanced immunosuppressive functions characterized by their high expression of effector molecules, such as CTLA4, TIGIT, ICOS, and GITR, and their ability to produce high amounts of anti-inflammatory cytokines, including IL-10 (Shevyrev & Tereshchenko, 2019).

Transcriptomic analysis has shown that numerous genes are differentially expressed during effector differentiation of Treg cells, and IRF4 appears to be one of the key transcription factors that drives the establishment of eTreg cell transcriptional program as well as the expression of major eTreg cell molecules, such as ICOS, IL-10, IL1rl1, Fgl2, and CCR8 (Zheng et al., 2009). In addition to IRF4, several other transcription factors, such as Blimp1, Myb, NF-kb, JunB, TCF1,

and LEF1 have been implicated in regulating the eTreg cell program (Cretney et al., 2011; Dias et al., 2017; Grinberg-Bleyer et al., 2017; Koizumi & Ishikawa, 2019; Koizumi et al., 2018; Yang et al., 2019).

eTreg cells generated in lymphoid organs can further migrate to targeted tissues, adapt to the specific tissue environments, and exert distinct suppressive functions that control various immunological or non-immunological processes such as autoimmunity, allergy, anti-tumor immunity, tissue homeostasis, and repair (Cretney et al., 2013).

In the last two decades, substantial efforts have been put into identifying novel molecular mechanisms necessary for proper Treg cell differentiation and function. Among them, miRNAs have been identified as crucial mediators of these processes. miRNAs are involved in regulating the generation, differentiation, activation, and function of various immune cells and their impacts on immune regulations will be discussed below.

1.2 MicroRNA in Immune Regulation

MiRNAs are a class of endogenous non-coding RNAs of ~21-25 nucleotides in length and play important roles in the post-transcriptional regulation of gene expression (Bushati & Cohen, 2007; He & Hannon, 2004). MiRNAs negatively regulate gene expression at the transcript or protein level in both animals and plants and are critical mediators in various cellular processes such as cell development, differentiation, growth, metabolism, and cell death (He & Hannon, 2004).

MiRNAs are encoded by genomic DNA and are generally transcribed by RNA polymerase II into a long primary transcript (known as pri-RNA) with a local hairpin structure where miRNA sequences are embedded. The pri-RNA is sequentially processed by RNase-III enzyme Drosha

into an approximately 70-nucleotide long stem-loop precursor miRNA (pre-miRNA), transported from the nucleus to the cytoplasm, and further processed by Dicer into a small, imperfect dsRNA duplex (miRNA:miRNA*) that contains both the mature miRNA and its complementary strand (miRNA*). The mature miRNA with a less stable 5' end is then preferentially loaded onto the protein Argonaute (Dunand-Sauthier et al.), the core component of the RNA-induced silencing complex (RISC) (Bartel, 2004; He & Hannon, 2004). The binding of the mature miRNA to AGO leads to a conformational change that exposes the conserved seed sequence, which is mostly situated at positions 2-7 from the miRNA 5' end. This sequence guides the RISC to specific target mRNAs, usually through the direct binding of miRNA seed sequence to the 3' untranslated regions (UTRs) of the target mRNAs and eventually results in the repression of protein expression or the promotion of mRNA degradation (Bartel, 2009).

Since the discovery of the pioneer miRNA lin-4 in *C. elegans* (Lee et al., 1993), hundreds of miRNAs have been identified and carefully studied for their key functions across different species under diverse biological contexts, including controls of leaf development in plants, neuronal patterning in nematodes, and modulation of hematopoietic lineage differentiation in mammals (He & Hannon, 2004). Considering their pivotal roles in orchestrating gene networks, the expression pattern of miRNAs is under tight temporal and spatial control. Dysregulation of miRNAs is associated with many human diseases, particularly cancer and immune disorders (Hayes et al., 2014; O'Connell et al., 2010).

Importantly, miRNA control has emerged as a critical regulatory component for the development, maintenance, and proper functions of the mammalian immune system. It was first demonstrated in hematopoietic stem cells (HSC) that overexpression of miR-181 in HSCs affected B-lymphopoiesis in vivo upon HSC transplantation, indicating the importance of dynamic miRNA

regulation during early hematopoiesis and lineage commitment (Chen et al., 2004; Lindsay, 2008). The miRNA network is also essential for B cell differentiation and functions. Ablation of the miRNAs through deficiency of Ago2 or Dicer leads to a substantial defect in B cell differentiation and a compromised antibody repertoire (Koralov et al., 2008; O'Carroll et al., 2007). For instance, miR-155 deficient B cells show an impaired capability to differentiate into germinal center cells and are unable to undergo Ig class switching (Thai et al., 2007; Vigorito et al., 2007). In addition, during T cell development, deletion of Dicer in immature thymocytes (referred to as "double-negative stage") results in a tenfold reduction of total thymocyte numbers, as well as in a defect in the positive selection process at a later time point between the double-positive and single-positive stage (Cobb et al., 2005; Lu & Liston, 2009; Xiao & Rajewsky, 2009). Moreover, the generation of different T helper lineages and the maintenance of their effector functions are also modulated by distinct miRNA expression patterns. For example, miR-155 promotes skewing towards T helper 1 (Th1) cells through repressing macrophage-activating factor (MAF) (Rodriguez et al., 2007). MiR-24 and miR-27 from the miR-23~27~24 clusters collaboratively limit Th2 responses through inhibiting IL-4 and GATA3 (Cho et al., 2016). In contrast to miR-23 and miR-27, enforced expression of miR-24 promotes the differentiation of Th1 and Th17, implying that individual miRNA from the same families can antagonize each other to fine-tune the biological effects of their regulation (Cho et al., 2016). In general, these findings demonstrate an intricate regulatory network of miRNAs to concert the immune cell development and their functions during different immune responses. A more detailed understanding of specific miRNAs in the context of particular types of immune settings might shed light on the molecular mechanisms of their regulation, and allow us to develop new approaches for disease diagnosis and treatment.

1.3 MicroRNAs in Regulatory T Cells

A critical role of miRNAs in Treg cells was initially discovered in *Dicer* or *Drosha* knockout (KO) mice, with ablations of these two key miRNA processing enzymes in Treg cells leading to severe and even fatal autoimmunity (Liston et al., 2008; Zhou et al., 2008). Consistent with these findings, the roles of individual miRNAs in regulating Treg cell development and functions were gradually uncovered. MiR-142 was demonstrated to be involved in mediating Treg cell suppressor capacity, as miR-142 deficient mice exhibit reduced Treg cell numbers, accompanied by increased frequencies and numbers of Th1 cell responses in the periphery (Huang et al., 2009). In addition, it was shown that miR-146a, prevalently expressed in Treg cells, is essential for their capacity to suppress IFNγ-dependent pathogenic Th1 and the associated inflammatory response through targeting of Stat1 (Lu et al., 2010). A recent study from our lab discovered that proper regulation of miR-27 is pivotal to safeguard Treg cell-mediated immunological tolerance. Excessive miR-27 expression impairs both thymic Treg development and peripheral Treg homeostasis, as well as their ability to control effector T cell activation and functions (Cruz et al., 2017).

MiR-155, an indispensable miRNA necessary for T- and B-cell maturation and innate immune responses, is also essential for Treg cell homeostasis (Burocchi et al., 2015; Lu et al., 2009; O'Connell et al., 2007; Rodriguez et al., 2007). Previously, Lu et al. demonstrated the cell-intrinsic mechanism through which miR-155 promotes Treg cell fitness in a Foxp3-dependent manner. Foxp3 directly regulates and maintains the high expression of miR-155, which targets the suppressor of cytokine signaling 1 (SOCS1), ensuring increased sensitivity of Treg cells to the essential growth factor IL-2 (Lu et al., 2009). Further study confirmed that miR-155 mediates SOCS1 repression in conferring Treg cell competitive fitness, by using $SOCS1^{KI}$ mice in which

the putative miR-155 target site of the 3'UTR of SOCS1 was mutated (Lu et al., 2015). Noticeably, in contrast to miR-155 KO mice, Treg numbers were not affected in $SOCS1^{KI}$ mice, which suggests the involvement of additional targets in the miR-155-dependent Treg cell maintenance(Lu et al., 2015; Lu et al., 2009). Moreover, since the understanding of miR-155's function in Treg cells relied on the analysis of mice with germline deficiency of miR-155, the altered function of other non-Treg cell populations with miR-155 deficiency could also contribute to the impaired Treg cell development and maintenance.

Furthermore, given that an intricate miRNA network integrates multiple pathways in Treg cell development, homeostasis, and function, the complexity and the severity of the disease phenotypes in mice harboring Treg cells devoid of miRNA cannot be attributed entirely to the loss of the aforementioned individual miRNAs, thus additional miRNAs essential for controlling other features of Treg cell biology require to be further characterized and elucidated.

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CHAPTER II: MIR-155 PROMOTES T REG CELL DEVELOPMENT BY SAFEGUARDING MEDULLARY THYMIC EPITHELIAL CELL MATURATION

2.1 Introduction

Regulatory T (Treg) cells constitute a specialized tolerogenic subset of cells recognized for maintaining immune homeostasis and preventing inappropriate reactivity to self-antigens and innocuous foreign-antigens (Josefowicz, Lu, et al., 2012). While Treg cells can be generated in the periphery and play a non-redundant role in restraining allergic-type inflammation at mucosal interfaces, those generated in the thymus (tTreg cells) are absolutely critical for controlling systemic and tissue-specific autoimmunity (Josefowicz, Niec, et al., 2012). To this end, the thymic medulla represents a specific site for establishing self-tolerance via the generation of tTreg cells in addition to its known role in mediating negative selection (Hinterberger et al., 2010). In the thymic medulla, medullary thymic epithelial cells (mTECs) express high levels of MHC II molecules, tissue-restricted antigens, and co-stimulatory ligands CD80/CD86 in order to foster an instructive cross-talk between these specialized thymic stromal cells and developing thymocytes (Lucas et al., 2016). Disruption of the aforementioned interactions results in failed de novo generation of tTreg cells (Aschenbrenner et al., 2007; Malchow et al., 2016; Salomon et al., 2000; Tai et al., 2005). The fact that loss of mTECs leads to an explicit defect in the tTreg cell compartment while leaving conventional CD4 single positive (CD4SP) thymocytes unaffected further substantiates an indispensable role for the thymic medulla in tTreg cell differentiation (Cowan et al., 2013).

To support the generation of tTreg cells, mTECs themselves need to differentiate properly. Activation of the RelB-dependent non-canonical NFκB pathway driven by tumor necrosis factor superfamily (TNFSF) cytokines such as RANKL, CD40L, and LTβ have been shown to be

essential for mTEC progenitors to undergo a stepwise differentiation process to generate immature MHCII^{lo}CD80^{lo} mTECs before mature MHCII^{hi}CD80^{hi} mTECs (Akiyama et al., 2008; Hikosaka et al., 2008; Irla et al., 2008). Among them, RANKL stimulation is particularly important for the induction of AIRE, a transcription factor that plays a major role in driving the expression of TRAs in mature mTECs (Anderson et al., 2002; Rossi et al., 2007; Zuklys et al., 2000). On the other hand, TGFβ has been shown to play a negative role in restraining mTEC maturation by interfering with the non-canonical NF-κB pathway (Hauri-Hohl et al., 2014). However, because TGFβ can be detected in the thymus shortly after birth, and predominantly in the thymic medulla (Konkel et al., 2014), exactly how mTECs shield themselves from persistent TGFβ exposure remains unclear (Hauri-Hohl et al., 2014).

MircoRNAs (miRNAs) comprise a class of small non-coding RNAs that regulate gene expression at the post-transcriptional level and whose roles in controlling the development and function of T cells, including Treg cells, are well established (Chong et al., 2008; Cobb et al., 2006; Cobb et al., 2005; Liston et al., 2008; Zhou et al., 2008). It is also now appreciated that miRNAs can regulate thymic T cell differentiation by maintaining a proper thymic microenvironment, where deletion of the miRNA network within TECs severely compromises thymic infrastructure and largely impacts mTECs (Khan et al., 2014; Papadopoulou et al., 2011; Zuklys et al., 2012). Nevertheless, the current understanding of individual miRNAs crucial for controlling different aspects of TEC biology and, more importantly, their impact on thymic T cell development, remains limited (Khan et al., 2015). Here, we show that miR-155, a prominent miRNA known for its diverse functions in various immune cell populations (Vigorito et al., 2013), plays an equally important role in mTECs. Previously, we and others have shown that elevated expression of miR-155 driven by Foxp3 ensures proper Treg cell homeostasis by maintaining their competitive fitness

(Lu et al., 2009). Our current work further demonstrates that miR-155 promotes Treg cell development in the thymus by safeguarding mTEC maturation. Mechanistically, RANK signaling induces miR-155 expression in the thymic medulla to alleviate the negative effects that ensue from the continuous presence of intrathymic TGFβ via targeting multiple known and previously uncharacterized molecules within this cytokine-signaling pathway. As such, the miR-155-TGFβ axis maintains the mature mTEC population and, thus, establishes a thymic microenvironment favorable for tTreg cell development.

2.2 Results

2.2.1 miR-155 promotes thymic Treg cell development in both T cell-intrinsic and -extrinsic manners.

miRNAs play a pivotal role in controlling multiple aspects of Treg cell biology. Among the many miRNAs predominantly expressed in Treg cells, miR-155 is crucial for promoting optimal Treg cell development and homeostasis partly through ensuring responsiveness to IL-2, a cytokine required for thymic and peripheral Treg cell maintenance (Kohlhaas et al., 2009; Lu et al., 2009). Mice harboring a germline deficiency of miR-155 present with diminished Treg cell numbers and frequencies, plus, additional studies utilizing mixed bone marrow (BM) chimeras have identified a cell autonomous role of miR-155 in controlling Treg cell biology (Kohlhaas et al., 2009; Lu et al., 2009). Consistent with this notion, upon T cell-specific miR-155 ablation (T-cKO; Fig. 2.6A), reduced frequencies as well as total numbers of Treg cells in the thymus are also detected (Fig. 2.1A-C). Interestingly, though, the degree of reduction in T-cKO mice does not fully recapitulate that observed in mice completely devoid of miR-155 (~30% in T-cKO *vs.* ~60% in miR-155 null; Fig. 2.1D) (Kohlhaas et al., 2009; Lu et al., 2009; Sánchez-Díaz et al., 2017). These

results suggest that loss of miR-155 expression in other non-T cell populations may also contribute to the impaired tTreg cell phenotype observed in mice containing miR-155 germline deficiency.

2.2.2 miR-155 is preferentially expressed in mature mTECs and is induced by RANKL stimulation.

mTECs function as a key stromal cell population crucial for the generation of Treg cells in the thymus, and so it is possible that miR-155 promotes tTreg cell development by regulating mTEC biology. In support of this notion, a previous miRNA profiling study has demonstrated that miR-155 is expressed at elevated levels in mTECs compared to cortical thymic epithelial cells (cTECs), another population of thymic stromal cells critical for early thymocyte development (Khan et al., 2015). In line with this work, our analyses of different thymic resident cell subsets also revealed higher expression levels of miR-155 in mTECs relative to cTECs, albeit lower than that in $CD45^{+}$ immune cells (Fig. 2.2A and B). Moreover, in scrutiny of two major subsets within the mTEC population, $CD80^{lo}MHCII^{lo}$ and $CD80^{hi}MHCII^{hi}$ (Fig. 2.2C), with the latter comprising the more mature subset essential for tTreg cell generation, we found that miR-155 expression is restricted to the $CD80^{hi}MHCII^{hi}$ mTEC compartment. Together, these data point to a potential role of miR-155-mediated regulation of mTEC maturation.

Among different TNFSF members that have been characterized in mTEC development, RANKL-RANK is the foremost determinant of mTEC development (Akiyama et al., 2008). To examine whether elevated miR-155 expression in mature mTECs is induced by RANK signaling, primary $CD80^{lo}MHCII^{lo}$ immature mTECs were isolated and stimulated with RANKL. Along with prior work (Rossi et al., 2007), we noted an induction of AIRE mRNA in these cells as early as 6hr following RANKL stimulation (Fig. 2.2E). Moreover, along with upregulation of AIRE, an

increase in the primary transcript of miR-155 (*pri-miR-155*) was also readily detectable (Fig. 2.2E). Contrarily, a reduction in *pri-miR-155* was observed concomitantly with diminished expression of *Aire* in mTECs isolated from mice treated with RANKL blocking antibodies (Fig. 2.2F). Collectively, these results locate RANK signaling in driving miR-155 expression in mTECs and imply functional relevance for miR-155 during mTEC development.

2.2.3 Expression of miR-155 in TECs is required to maintain optimum mTEC maturation and tTreg cell development.

To investigate the potential role of miR-155 in mTEC maturation and its subsequent impact on tTreg cell development, we generated mice with TEC-specific ablation of miR-155 (TEC-cKO) by crossing miR-155 floxed mice (*miR-155*^{fl}) to FOXN1-Cre mice (Fig. 2.6B-D). Considering that deletion of miR-155 is not restricted to mTECs but rather encompasses the entire thymic epithelium, we first sought to determine whether the cellularity and phenotype of the thymic epithelia would be impacted by the loss of miR-155. As depicted in Fig. 2.3A (and data not shown), we did not detect any alterations in total thymic cellularity, including proportions of cTECs and mTECs, upon deletion of miR-155 in TECs relative to control WT or T-cKO mice. In contrast, we found that the frequency of CD80^{hi}MHCII^{hi} mature mTECs was significantly reduced in TEC-cKO mice in comparison to both WT and T-cKO controls, supporting our proposed function of miR-155 during mTEC maturation (Fig. 2.3B). Accompanied by a decrease in mature mTECs which also express AIRE, we detected an increase in the frequency of a subset of AIRE^{-}CCL21^{+} mTECs in TEC-cKO mice (Fig. 2.7A). Unlike AIRE-expressing mTECs, the development of this specific mTEC subset relies on LTβ receptor signaling and has been implicated in recruiting (and/or retaining) positively selected CCR7^{+} thymocytes to the medulla (Lkhagvasuren et al., 2013;

Zhang & Bhandoola, 2014). On the other hand, no significant change was observed in DCLK1-expressing thymic tuft cells (Fig. 2.7B), another mTEC subset that is transcriptionally distinct from the AIRE$^+$ mature mTECs (Bornstein et al., 2018). Interestingly, despite the reduction of mature mTECs in TEC-cKO mice, on a per-cell basis, levels of AIRE in mature mTECs were comparable between TEC-cKO and control mice (Fig. 2.7C and D), implying that miR-155 does not directly regulate AIRE expression despite its role in mTEC maturation.

Due to the aforementioned role of mature mTECs in tTreg cell development and the impairment in mature mTECs from mice bearing TEC-specific ablation of miR-155, we next asked whether the generation of tTreg cells is similarly affected in TEC-cKO mice. Analogous to what has been discerned in T-cKO mice, a comparable reduction in tTreg cell frequency was detected in TEC-cKO mice (Fig. 2.3C), highlighting that miR-155 as expressed in T cells and TECs contributes equally to Treg cell development in the thymus. Further supporting this notion, a similar reduction in the frequency of nascent tTreg cells in TEC-cKO mice was also seen when mature recirculating CD73$^+$ Treg cells were excluded from the total tTreg cell population (Fig. 2.3D and E). It should be noted that while miR-155 deletion in mTECs results in a reduction in tTreg cell frequency, the proliferative capacity as well as expression levels of Foxp3 and other Treg cell-associated molecules were unaffected in these Treg cells relative to WT controls (Fig. 2.8). In the spleen, even though the frequency of Treg cells in TEC-cKO mice remained lower than WT controls, significantly more Treg cells in the spleen of TEC-cKO mice were seen when compared to T-cKO mice (Fig. 2.3F). While the difference between the results obtained from the thymus and the spleen is intriguing, it is not surprising seeing as miR-155 is a known crucial regulator conferring competitive fitness to Treg cells both in the thymus and in the periphery in a cell autonomous manner (Lu et al., 2009). Taken together, these data clearly demonstrate an

indispensable role of TEC-derived miR-155 in controlling mTEC maturation and, subsequently, the generation of Treg cells in the thymus.

2.2.4 miR-155 limits TGFβ signaling via targeting multiple components of the TGFβ signaling pathway.

The TGFβ signaling cascade has previously been reported to play a regulatory role in limiting the establishment and function of the thymic medulla, specifically by influencing the differentiation of mTECs (Hauri-Hohl et al., 2014). In addition, miR-155 has also been implicated in regulating TGFβ signaling by directly targeting *Tgfbr2* and *Smad2* in human lung fibroblasts and in THP-1 monocyte cell lines, respectively (Chu, 2017; Louafi et al., 2010). Therefore, it is possible that miR-155 also promotes mTEC maturation by restraining TGFβ signaling. Indeed, we observed increased levels of both *Tgfbr2* and *Smad2* in mature mTECs isolated from TEC-cKO mice, indicating that these two genes are also subject to regulation by miR-155 in mature mTECs (Fig. 2.4A and B). Consistently, we detected higher levels of *P21*, an established TGFβ-induced gene (Datto et al., 1995), in mature mTECs devoid of miR-155. The levels of *Myc*, a gene known to be repressed by TGFβ activity (Frederick et al., 2004), were, alternatively, reduced (Fig. 2.4C and D). Overall, these results are suggestive of enhanced TGFβ signaling in the absence of miR-155-mediated gene regulation.

Since miRNAs usually exert their regulatory effects by targeting multiple genes in a shared pathway or protein complex to ensure biological impact (Ebert & Sharp, 2012), we sought to define additional targets in the TGFβ signaling pathway that may be controlled by miR-155 in mature mTECs. After analyzing previous results obtained from high-throughput sequencing of RNAs isolated by cross-linking immunoprecipitation (HITS-CLIP), a biochemical approach that affords

the identification of functional miRNA-mRNA interaction in a given tissue/cell samples (Loeb et al., 2012), we identified SMAD3, a molecule that acts cooperatively with SMAD2 to form major TGFβ signaling transducers, as another potential miR-155 target (Fig. 2.4E and F). Supporting this notion, our luciferase reporter studies confirmed that miR-155 directly represses SMAD3 (Fig. 2.4G), and mature mTECs isolated from TEC-cKO mice express significantly higher amounts of *Smad3* transcript (Fig. 2.4H). Moreover, by taking similar approaches, we also identified and confirmed RNF111, as a direct target of miR-155 (Fig. 2.4E-H). RNF111 (or "Arkadia") is an E3 ubiquitin ligase recognized for its role in enhancing TGFβ responses by promoting the degradation of c-SKI, a known negative regulator of TGFβ signaling that blocks TGFβ-driven transcriptional activation and repression by forming an inhibitory complex with SMAD proteins (Sharma et al., 2011; Suzuki et al., 2004). Thus, our data show that through targeting multiple requisite components within the TGFβ signaling pathway, ranging from the receptor, to major signal transducers, and to the E3 ligase that degrades the TGFβ signaling inhibitor, miR-155 acts as a key molecule involved in attenuating TGFβ signaling in mTECs amid their maturation process.

2.2.5 Diminished mTECs and tTreg cells in TEC-cKO mice are largely rescued by partial TGFβR deletion.

While our results demonstrate that TEC-specific miR-155 ablation leads to enhanced TGFβ signaling in mTECs, it remains obscure as to whether loss of miR-155-dependent regulation of TGFβ signaling is responsible for the mTEC and tTreg cell phenotypes observed in TEC-cKO mice. Previously, it has been shown that short-term systemic pharmacological inhibition of TGFβ signaling with the TGFβRI kinase inhibitor selectively increases the number of mature mTECs (Hauri-Hohl et al., 2014). Interestingly, however, such treatment did not seem to rescue the

defective tTreg cell phenotype observed in mice with TEC-specific miR-155 ablation; only a modest increase of tTreg cell frequencies was detected in TEC-cKO mice following treatment of the TGFβRI kinase inhibitor compared to the untreated group (Fig. 2.5A and B). It should be noted that TGFβ signaling in thymocytes has previously been reported to be needed for both induction of Foxp3 and the differentiation of tTreg cells (Konkel et al., 2014). It is therefore possible that any positive effect resulting from blockade of TGFβ signaling in mTECs is masked by the loss of TGFβ-driven tTreg cell induction in mice with systemic TGFβRI kinase inhibitor administration.

To directly examine the role of the miR-155-TGFβ signaling axis in mTECs and its subsequent impact on tTreg cell development, we opted for a genetic approach by removing one allele of *Tgfbr2* specifically in the thymic epithelia of TEC-cKO mice (TEC-cKO/*Tgfbr2*$^{fl/+}$). As shown in Fig. 2.5C and D, a significantly enlarged mature CD80^{hi}MHCII^{hi} mTEC population was seen in TEC-cKO/*Tgfbr2*$^{fl/+}$ mice compared to TEC-cKO mice. Accordant with the previously established role of mature mTECs in driving tTreg cell development, substantially augmented tTreg cell frequencies were also detected in accompany with an increase in the mature mTEC population in TEC-cKO mice containing *Tgfbr2* heterozygosity (Fig. 2.5E and F). Notably, the frequencies of mature mTECs and tTreg cells in TEC-cKO/*Tgfbr2*$^{fl/+}$mice remained lower than those in WT controls (Fig. 2.5C-F), indicating that additional miR-155 targets independent of TGFβ signaling might also contribute to the mTEC and tTreg cell phenotypes observed in TEC-cKO mice. Nevertheless, our data effectively demonstrate that miR-155 supports mTEC maturation and the resultant tTreg cell development by restricting TGFβ signaling.

2.3 Discussion

The thymus is a specialized organ where T cells develop. In addition to the production of Tconv cells that serve as a major arm of the adaptive immune system, the generation of Treg cells in the thymus is crucial for establishing immunological tolerance and for preventing fatal autoimmune diseases. Within the thymic microenvironment, the thymic medulla has previously been shown to be instrumental for the development of Treg cells (Akiyama et al., 2014; Cowan et al., 2013; Hauri-Hohl et al., 2014). Disruption of mTEC maturation by inhibiting RANK signaling results in a significant defect in tTreg cell generation, while elimination of TGFβ signaling facilitates mTEC maturation and leads to increased frequencies and absolute numbers of tTreg cells. In this study, we locate a previously uncharacterized role of miR-155 in controlling mTEC maturation. Through targeting the TGFβ signaling pathway, our data demonstrate that miR-155, a miRNA whose expression in mTECs is induced upon RANKL stimulation, ensures proper mTEC maturation and, consequently, the optimal production of tTreg cells.

Since the discovery of miRNAs and their roles in regulating the development and function of the immune system, miR-155 has attracted a lot of attention due to its unique ability to shape the transcriptome of a wide variety of both innate and adaptive immune cells in order to control diverse biological functions, ranging from immune activation to memory formation (Vigorito et al., 2013). In Treg cells, expression of miR-155 is essential for maintaining normal Treg cell frequencies both in the thymus and periphery (Kohlhaas et al., 2009; Lu et al., 2009). The reduction in Treg cell numbers observed in mice devoid of miR-155 could be partly attributed to attenuation of IL-2 signaling in the absence of miR-155-mediated regulation of SOCS1 (Lu et al., 2015; Lu et al., 2009). However, even though the cell-intrinsic role of miR-155 in Treg cells has been well established (Kohlhaas et al., 2009; Lu et al., 2009), considering the pleiotropic functions of miR-

155 in many other cell types, it has long been speculated that miR-155 may regulate Treg cell development and homeostasis in a Treg cell-extrinsic manner (Lu et al., 2015). Supporting this notion, our analyses of TEC-cKO mice clearly demonstrate a non-autonomous role for miR-155 in tTreg cell development through selective support of mTEC maturation. Interestingly, numerically speaking, it seems that combining the loss of miR-155 in T cells and TECs largely accounts for the tTreg cell phenotype in mice with the germline miR-155 deletion. Nevertheless, as miR-155 deficiency is already known to diminish the antigen-presenting and costimulatory capacities of DCs (Rodriguez et al., 2007), and since both mTECs and DCs are crucial for optimal tTreg cell generation (Perry et al., 2014; Proietto et al., 2008), the possibility that impaired DC function also contributes to the reduction in Treg cell numbers seen in miR-155 null mice cannot be completely excluded.

It has been previously reported that increased active TGFβ in the thymus can be detected shortly after birth in response to increasing thymocyte apoptosis (Konkel et al., 2014), In particular, enrichment of TGFβ activity has been observed in the thymic medulla with the direct effect of TGFβ on developing thymocytes having been implicated to be vital for the differentiation of tTreg cells, albeit dispensable for their maintenance (Konkel et al., 2014; Liu et al., 2008). However, considering the aforementioned role of TGFβ in limiting the establishment of the thymic medulla (Hauri-Hohl et al., 2014), it was perplexing at first as to how mTECs could be counteracting the negative effects of TGFβ on their maturation and expansion. Moreover, as TGFβ signaling in mTECs was shown to negatively impact tTreg cell generation (Hauri-Hohl et al., 2014), it also seems counterintuitive and counterproductive for intrathymic TGFβ to possess such dual yet contrasting roles in tTreg cell development. To this end, our current study demonstrates that expression of miR-155 in mTECs is rapidly induced upon RANKL stimulation and remains

high in the mature mTEC population. On the other hand, in the thymocytes, elevated expression of miR-155 could only be detected in Foxp3$^+$ tTreg cells as reported in our previous work (Lu et al., 2009). This cell-type specific miR-155 expression pattern could thus potentially permit mTEC-dependent tTreg cell differentiation through miR-155-mediated targeting of the TGFβ signaling pathway in mTECs, while allowing for TGFβ-driven Foxp3 induction in developing thymocytes where miR-155 is minimally expressed. Consistent with this notion, in our studies, the tTreg cell phenotype observed in mice with TEC-specific miR-155 ablation could only be rescued by tuning down TGFβ signaling through selective partial deletion of the TGFβ receptor in TECs, whereas modest effects were detected when TGFβ signaling was non-specifically blocked by systemic administration of TGFβ inhibitor.

Previously, it has been suggested that the thymic medulla is necessary for tTreg cell but dispensable for Tconv cell development, since the absence of RelB-dependent mTECs results in a selective loss of Foxp3$^+$ tTreg cells (Cowan et al., 2013). In support of this, our data shows that impaired mTEC maturation in mice with TEC-specific deletion of miR-155 leads to a specific reduction in tTreg cell frequencies while comparable numbers of CD4SP (and CD8SP) thymocytes are found in TEC-cKO and control mice. However, even though the Tconv cell compartment was seemingly untouched, it is likely this altered thymic medulla may also impact the T cell repertoire given that one of the major functions of mTECs is to enforce the negative selection of thymocytes. Indeed, it has been shown that in addition to elevated tTreg cell numbers, enhanced mTEC maturation and expansion in mice with complete deletion of TGFβ signaling in TECs results in a marked reduction in autoreactive T cell clones (Hauri-Hohl et al., 2014). Therefore, it is plausible that defective mTEC maturation in TEC-cKO mice may lead to impaired negative selection and, thus, an altered TCR repertoire, in addition to the observed tTreg cell phenotype. Interestingly,

however, no apparent signs of autoimmunity could be detected in our analyses of TEC-cKO mice. These results suggested that the remaining Treg cells (also likely having an altered TCR repertoire) are still capable of controlling autoreactivity in mice despite having potentially faulty negative selection. Consistent with this notion, even in mice with severely impaired mTEC maturation caused by the deletion of the entire miRNA pathway in TECs, no spontaneous autoimmunity could be observed (Khan et al., 2014; Zuklys et al., 2012). Nevertheless, further studies utilizing different autoimmune disease models or aged mice are needed to clarify the functional consequences resulting from miR-155 deficiency in the thymic medulla.

In the past decade, the role of miRNAs in controlling diverse immune responses has become a focal point of intense investigation. Still, despite our accumulating knowledge of miRNA-mediated gene regulation in different immune cell populations, relatively limited efforts have been spent on understanding how miRNAs impact the immune system by shaping the environmental cues supplied by non-immune cells. Here, our results demonstrate miR-155, a miRNA known for its role in promoting the competitive fitness and proliferative potential of Treg cells in a cell-intrinsic manner (Lu et al., 2009), also facilitates Treg cell development in the thymus by ensuring the proper maturation of the thymic medulla. Previously, we have shown that the same miRNA can impose its gene regulatory effect in different immune cell subsets, enabling them to play their specialized roles in producing a concerted response to a particular environmental stimulus (Cho et al., 2018). Our current work has further extended this concept and suggests that additional attention be paid to stromal cells residing in the same microenvironments in order to better understand the biological impact of a given miRNA on a selective immunological process.

2.4 Materials and Methods

Mice

miR-155^{fl} mice (Hu et al., 2014) were bred with CD4-Cre mice (Lee et al., 2001) and FOXN1-Cre mice (Gordon et al., 2007), respectively, to obtain mice with T cell-specific deletion (T-cKO) and TEC-specific deletion (TEC-cKO) of miR-155. *Tgfbr2*^{fl} mice (Levéen et al., 2002) were bred with TEC-cKO mice, to obtain TEC-cKO/*Tgfbr2*$^{fl/+}$ mice. Unless otherwise indicated, 5 to 6 weeks old mice were used. All mice were maintained and handled in accordance with the Institutional Animal Care and Use Guidelines of UCSD and National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and the ARRIVE Guidelines.

Tissue preparation

For T cell analysis, single-cell suspensions of thymus and spleen were prepared by slide mechanical disruption. TECs were isolated as previously described (Seach et al., 2013). In brief, thymus was mechanically disrupted and digested with DNase I (Sigma-Aldrich) and Liberase (Roche). The resulting single-cell suspension was filtered and washed once in MACS buffer. In some experiments, cells were used immediately for FACS analysis. Otherwise, density-gradient centrifugation was performed for TEC enrichment prior to FACS analysis or sorting. Briefly, cells were resuspended in 2 ml of 1.115 g/ml isotonic Percoll and overlaid with 1.5 ml of 1.065 g/ml isotonic Percoll, followed by a layer of 1 ml of PBS. Samples were then centrifuged at 600 g at 4 °C for 30 min with the brakes off. The thymic stroma accumulated between the top and middle layers, and was collected and washed for subsequent FACS analysis or cell sorting.

Flow cytometry and antibodies

For FACS analysis, cells were first stained with Ghost Dye Red 780 (Tonbo Biosciences) followed by surface antibody staining for CD4 (RM4-5), CD8a (53-6.7), CD45 (30-F11), EpCAM (G8.8), Ly51 (6C3), BiotinylatedUEA-1 (Vector Labs), CD80 (16-10A1), MHCII (M5/114.15.2), TCRβ□(H57-597), Streptavidin (eBioscience), CD44 (IM7), CD62L (MEL-14), CD25 (PC61), GITR (DTA-1). Intracellular staining for Foxp3 (FJK-16s), Ki67 (SolA15), AIRE (5H12), CTLA-4 (UC10-4B9), CCL21 (59106), DCLK1 (Abcam polyclonal ab31704) and AlexaFluor-488–conjugated Donkey anti-rabbit IgG Ab (Poly4064) was completed after fixation and permeabilization with Foxp3/Transcription Factor Staining Kit according to manufacturer protocol (Tonbo). Cells were fixed in 2% paraformaldehyde prior to analysis by flow cytometry. For Treg cell analysis, total tTreg cells were gated on $Foxp3^+$ cells from the $CD4^+CD8^-$ population. For nascent tTreg cells, $CD73^-$ cells were first gated from the $CD4^+CD8^-$ population, followed by $Foxp3^+$ gating. As for TEC analysis, cells were first gated/sorted from the $CD45^-EpCAM^+$ population, followed by using Ly51 and UEA-1 to separate cTECs ($Ly51^+UEA\text{-}1^-$) and mTECs ($Ly51^-UEA\text{-}1^+$). Within the mTECs, immature (or $mTEC^{lo}$) and mature (or $mTEC^{hi}$) mTECs were separated by the expression levels of CD80 and MHCII. Moreover, AIRE and CCL21 were used to identify the $CCL21^+AIRE^-$ mTEC subset while DCLK1 was used to label the thymic tuft cells. FACS Data were collected by BD LSRFortessa or BD LSRFortessa X-20 (BD Biosciences) and analyzed by FlowJo (Tree star). Finally, BD BD FACSAria Fusion (BD Biosciences) was used for cell sorting.

Quantitative PCR analysis

Different thymic cell subsets, $CD45^+$($CD45^+EpCAM^-$), cTEC ($CD45^-EpCAM^+Ly51^+UEA\text{-}1^-$), mTEC ($CD45^-EpCAM^+Ly51^-UEA\text{-}1^+$) cells, immature mTEC, ($CD45^-EpCAM^+Ly51^-UEA\text{-}1^+CD80^{lo}MHCII^{lo}$) and mature mTEC ($CD45^-EpCAM^+Ly51^-UEA\text{-}1^+CD80^{hi}MHCII^{hi}$) cells were sorted on a FACSAria Fusion (BD Biosciences) and then total mRNA was isolated using a miRNeasy kit (Qiagen) according to the manufacturer's instructions. To determine expression of miR-155, TaqMan MicroRNA Assay (Thermo Fisher Scientific) was performed. For other gene detection, cDNA was generated using iScript cDNA Synthesis Kit (Bio-Rad), followed by qPCR reactions using SYBR Green PCR Mix (Thermo Fisher Scientific). The primer sequences used were as follows: *Tgfbr2*: 5'-GAGAAGCCGCATGAAGTCTG-3' (F), 5'-CATGAAGAAAGTCTCGCCCG-3' (R); *Smad2*: 5'-ATATAGGAAGGGGAGTGCGC-3' (F), 5'-AAACGGCTTCAAAACCCTGG-3' (R); *Smad3*: 5'-ACTTGGACCTACAGCCAGTC-3' (F), 5'-TGCATTCCGGTTGACATTGG-3' (R); *Rnf111*: 5'-CAGCCTTCCACAGTGTCAGA-3' (F), 5'-GGTGTGCTAATGCATGATGG-3' (R); *Myc*: 5'-CACTCACCAGCACAACTACG-3' (F), 5'-GTTCCTCCTCTGACGTTCCA-3' (R); *P21:* 5'-GCAGATCCACAGCGATATCC-3' (F), 5'-CAACTGCTCACTGTCCACGG-3' (R); *Aire*: 5'-AGGTCAGCTTCAGAGAAAACCA-3' (F), 5'-TCATTCCCAGCACTCAGTAGA-3' (R); *pri-miR-155*: 5'-ACCCTGCTGGATGAACGTAG-3' (F), 5'-CATGTGGGCTTGAAGTTGAG-3' (R); *Gapdh*: 5'-CGTCCCGTAGACAAAATGGT-3' (F), 5'-TCAATGAAGGGGTCGTTGAT-3' (R); *pri-miR-29a*: 5'-AACCGATTTCAGATGGTGCT-3' (F), 5'-AAGCCTTCTCTGGAAGTGGAC-3' (R).

In vitro RANKL Stimulation

$CD80^{lo}MHCII^{lo}$ immature mTECs were sorted on a FACSAria Fusion (BD Biosciences) into complete RPMI, followed by stimulation with 500 ng/ml recombinant mouse RANKL (BioLegend) for 6 hrs at 37 °C.

In vivo mouse treatment

For *in vivo* RANKL blockade, anti-RANKL antibody (IK22/5) or isotype control antibody (RTK2758) was administrated to 4-week old mice at a dose of 250 μg in PBS every other day via intra-peritoneal (i.p.) injection for a total of 3 injections. Mouse thymi were collected for further study 1 day after the last injection. For systemic administration of TGFβRI kinase inhibitor, SB431542 (Selleckchem) (10mg per kg body weight, 0.2 mg in 100μL of 2% DMSO + 30% PEG300 + PBS) or vehicle control was administrated via i.p. injection once a day from day 1 to day 6 and analyzed 12 days after the first injection.

Luciferase reporter assay

The 3'UTR sequences of SMAD3 and RNF111 were amplified from mouse genomic DNA and cloned into pSiCheck2 vector (Promega). Site-direct mutagenesis (Agilent) was performed to obtain mutants of SMAD3 3'UTR and RNF111 3'UTR, respectively. Indicated 3'UTR WT or mutant plasmids were transfected into HEK293T (ATCC CRL-3216) cells along with either a miR-155-expressing plasmid or a control empty vector. Luciferase activity was determined by the Dual luciferase reporter assay system (Promega) according to the manufacture's instructions at 24 hr after transfection.

Statistical Analyses

An unpaired, 2-tailed Student's t test (or 1-way ANOVA for studies with more than two groups) was done on all reported data using Prism software (GraphPad) (*P < 0.05; **P < 0.01; ***P < 0.001; ns: not significant.). All experiments were performed independently at least three times to ensure the reproducibility of the data.

Chapter 2, in full, is an adapted version of the material published in The Journal of Experimental Medicine. **Dong, J.***, Warner, L. M.*, Lin, L. L., Chen, M. C., O'Connell, R. M., & Lu, L. F. (2021). miR-155 promotes T reg cell development by safeguarding medullary thymic epithelial cell maturation. J Exp Med, 218(2). * denotes co-first authorship. This dissertation author was the co-first author of the article.

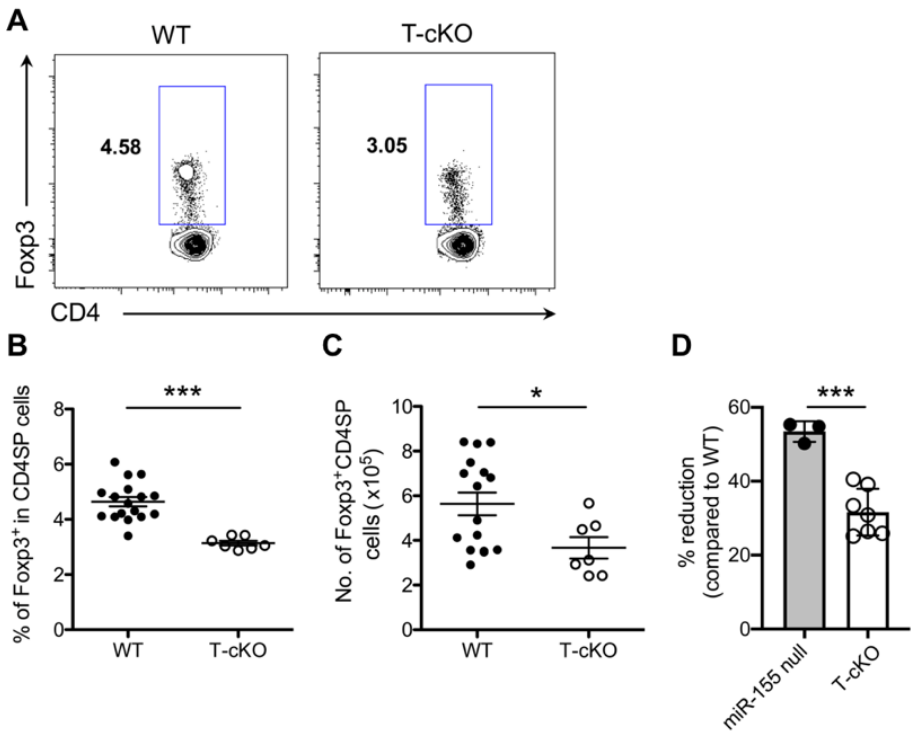

Figure 2.1 miR-155 promotes thymic Treg cell development in both T cell-intrinsic and –extrinsic manners. (A) FACS analysis, **(B)** frequencies and **(C)** absolute numbers of total Foxp3$^+$ Treg cells in the thymus of 5-6 weeks old WT and T-cKO mice. **(D)** Percentages of the reduction of tTreg cell frequencies (on the basis of corresponding WT controls) in miR-155 null and T-cKO mice. Each symbol represents an individual mouse, and the bar represents the mean. Data are pooled from at least three independent experiments. *, $P < 0.05$; ***, $P < 0.001$.

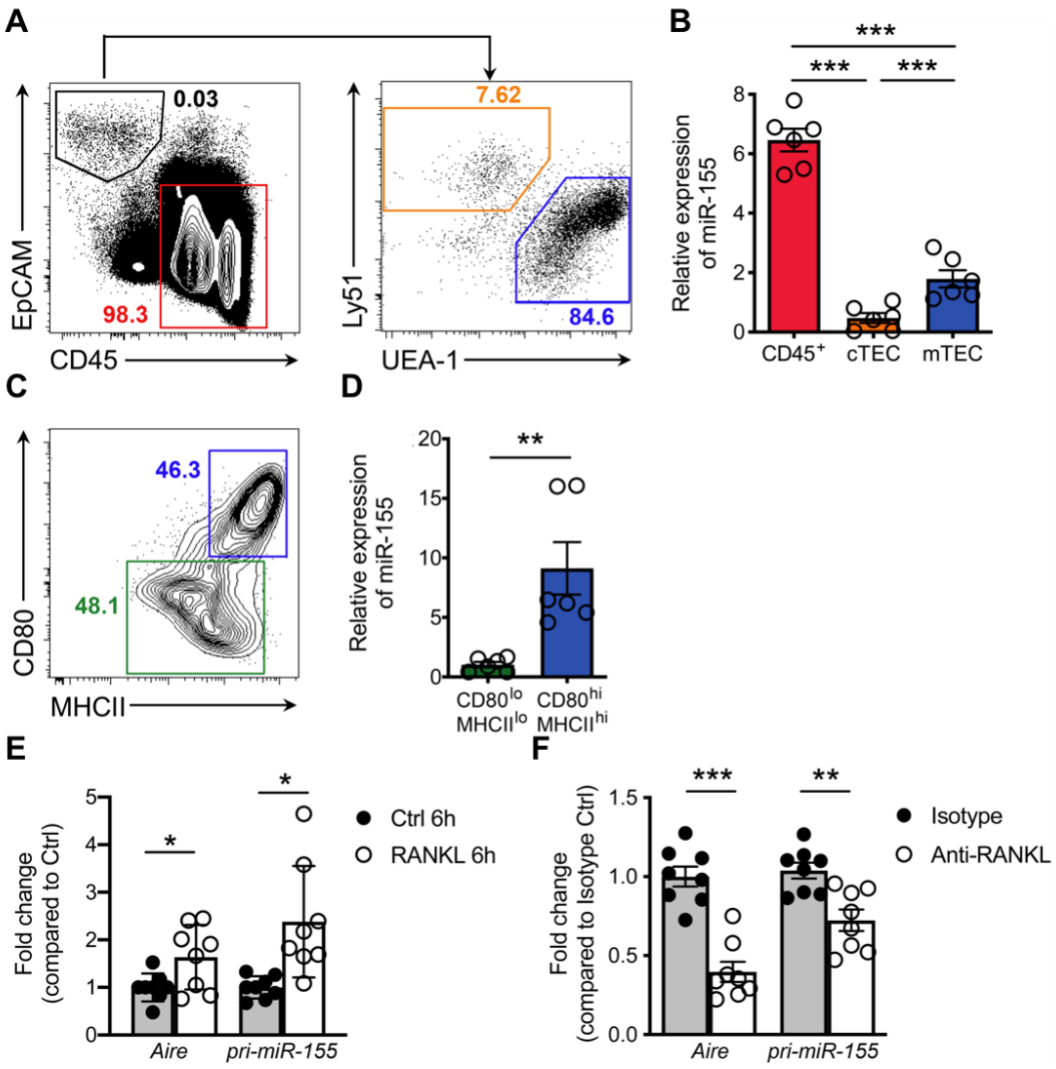

Figure 2.2 RANKL stimulation results in elevated miR-155 expression in mature mTECs. **(A)** Representative FACS profiles with gating strategy for different thymic subsets are shown. Hematopoietic cells were defined as $CD45^{+}EpCAM^{-}$, TECs were defined as $CD45^{-}EpCAM^{+}$ and further divided into cTEC ($Ly51^{+}UEA\text{-}1^{-}$) and mTEC ($Ly51^{-}UEA\text{-}1^{+}$) populations. **(B)** qPCR analyses of miR-155 expression in hematopoietic cells, cTECs and mTECs. **(C)** Immature and mature mTECs within the mTEC population were further discriminated based on the levels of expression of CD80 and MHCII molecules. **(D)** qPCR analyses of miR-155 expression in $CD80^{lo}MHCII^{lo}$ immature mTECs and $CD80^{hi}MHCII^{hi}$ mature mTECs. **(E)** qPCR analyses for *Aire* and *pri-miR-155* expressions in sorted primary immature mTECs stimulated with 500 ng/ml RANKL for 6 hours *in vitro* or **(F)** in total mTECs isolated from mice administrated with anti-RANKL or isotype control antibodies. The n-fold changes on the basis of corresponding untreated control groups were shown. Each symbol represents an individual mouse, and the bar represents the mean. Data are pooled from at least three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

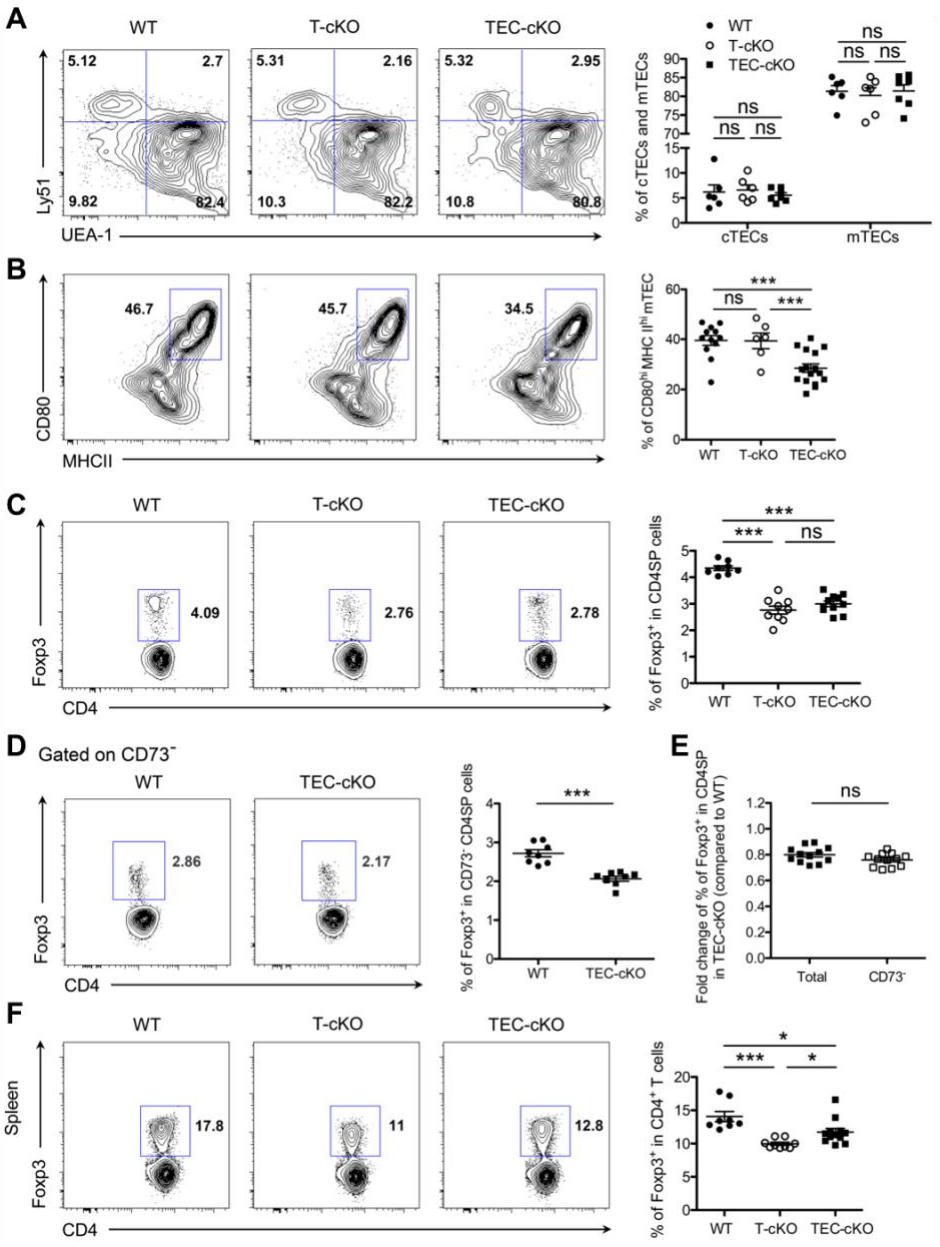

Figure 2.3 miR-155 deficiency in TECs leads to a diminished mature mTEC population and impaired thymic Treg cell development. (A) FACS analysis and frequencies of Ly51^{+}UEA-1^{-} cTEC and Ly51^{-}UEA-1^{+} mTEC populations from 5-6 weeks old WT, T-cKO, and TEC-cKO mice. **(B)** FACS analysis and frequencies of CD80^{hi}MHCII^{hi} mTECs from 5-6 weeks old WT, T-cKO, and TEC-cKO mice. FACS analysis and frequencies of **(C)** total Foxp3^{+} Treg cells or **(D)** CD73^{-} Foxp3^{+} Treg cells in the thymus of 5-6 weeks old WT, T-cKO, and TEC-cKO mice. **(E)** Fold change of % of total or CD73^{-} thymic Treg cells in TEC-cKO mice compared to WT littermates. **(F)** FACS analysis and frequencies of Foxp3^{+} Treg cells in the spleen of 5-6 weeks old WT and TEC-cKO mice. Each symbol represents an individual mouse, and the bar represents the mean. Data are pooled from at least three independent experiments. *, $P < 0.05$; ***, $P < 0.001$.

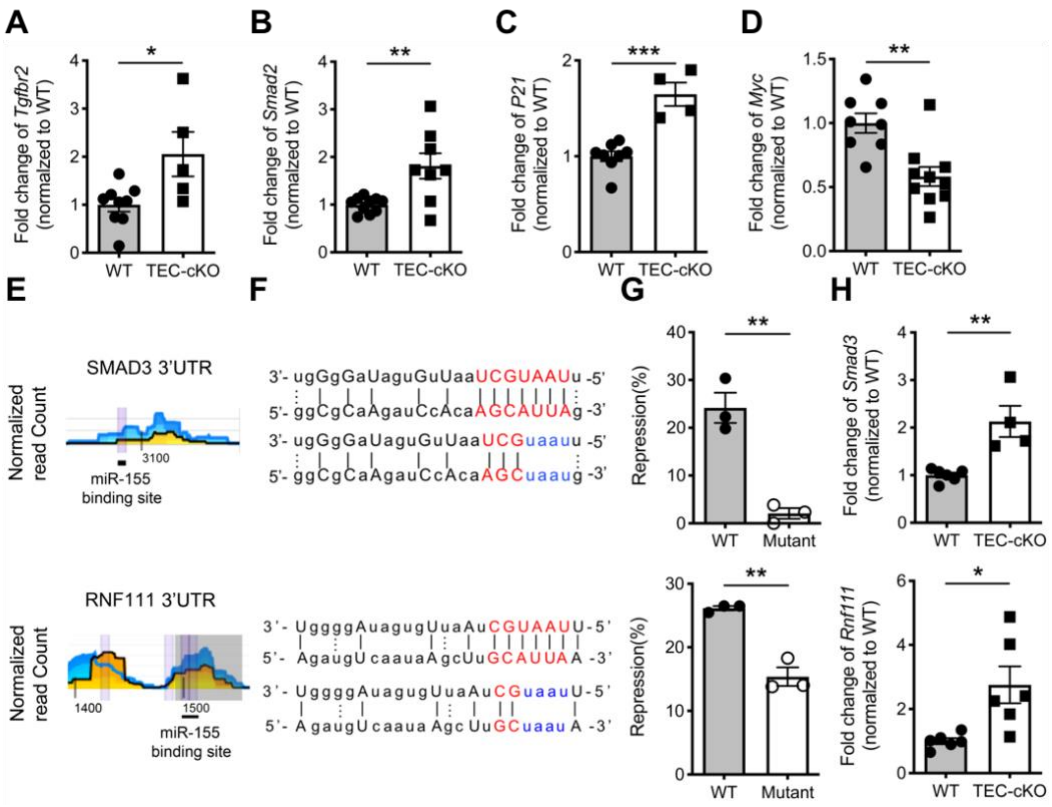

Figure 2.4 miR-155 regulates TGF-β signaling in mature mTECs via targeting multiple key components. qPCR analyses for the expressions of **(A)** *Tgfbr2*, **(B)** *Smad2*, **(C)** *P21* and **(D)** *Myc* in sorted CD80^{hi}MHCII^{hi} mature mTECs from 5-6 weeks old WT and TEC-cKO mice. The n-fold changes on the basis of each corresponding WT controls were shown. **(E)** HITS-CLIP analyses (the underlying numbers represent the necleotide position related to the start of the 3' UTR) and **(F)** sequence alignments of the putative miR-155 binding sites in 3'UTR of SMAD3 (Huppertz et al.) and RNF111(lower). Mutations of the corresponding miR-155 target sites are shown in blue. **(G)** Ratios of repressed luciferase activity of cells with SMAD3 3' UTR (Huppertz et al.) and RNF111 3' UTR (lower) with or without mutations in the seed sequences in the presence of miR-155 compared with cells transfected with empty vector. **(H)** qPCR analyses of the expressions of *Smad3* (Huppertz et al.) and *Rnf111* (lower) in sorted CD80^{hi}MHCII^{hi} mature mTECs from 5-6 weeks old WT and TEC-cKO mice. Each symbol represents an individual mouse, and the bar represents the mean. Data are pooled from at least three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

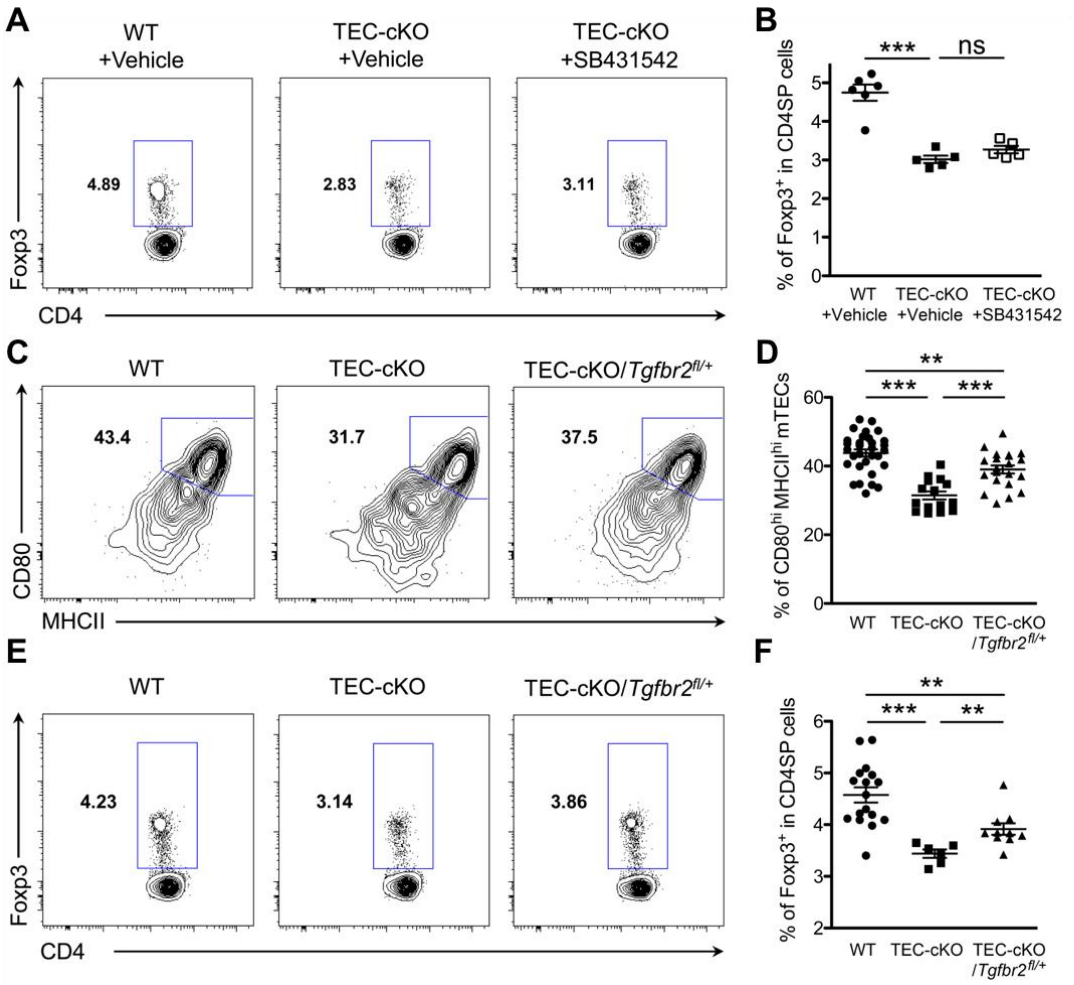

Figure 2.5 *Tgfbr2* heterozygosity in TECs restores mature mTEC and tTreg cell phenotypes in TEC-cKO mice. (A) FACS analyses and **(B)** frequencies of Foxp3^{+} Treg cells in the thymus of 6-8 weeks old WT and TEC-cKO mice treated daily with the TGFβRI kinase inhibitor SB431542 or vehicle only via intra-peritoneal injection from day 1 to day 6 and analyzed 12 days after the first injection. **(C)** FACS analysis and **(D)** frequencies of CD80^{hi}MHCII^{hi} mature mTECs in the thymus of 5-6 weeks old WT, TEC-cKO and TEC-cKO/*Tgfbr2*$^{fl/+}$mice. **(E)** FACS analysis and **(F)** frequencies of Foxp3^{+} Treg cells in the thymus of 5-6 weeks old WT, TEC-cKO and TEC-cKO/*Tgfbr2*$^{fl/+}$mice. Each symbol represents an individual mouse, and the bar represents the mean. Data are pooled from at least three independent experiments. **, P < 0.01; ***, P < 0.001.

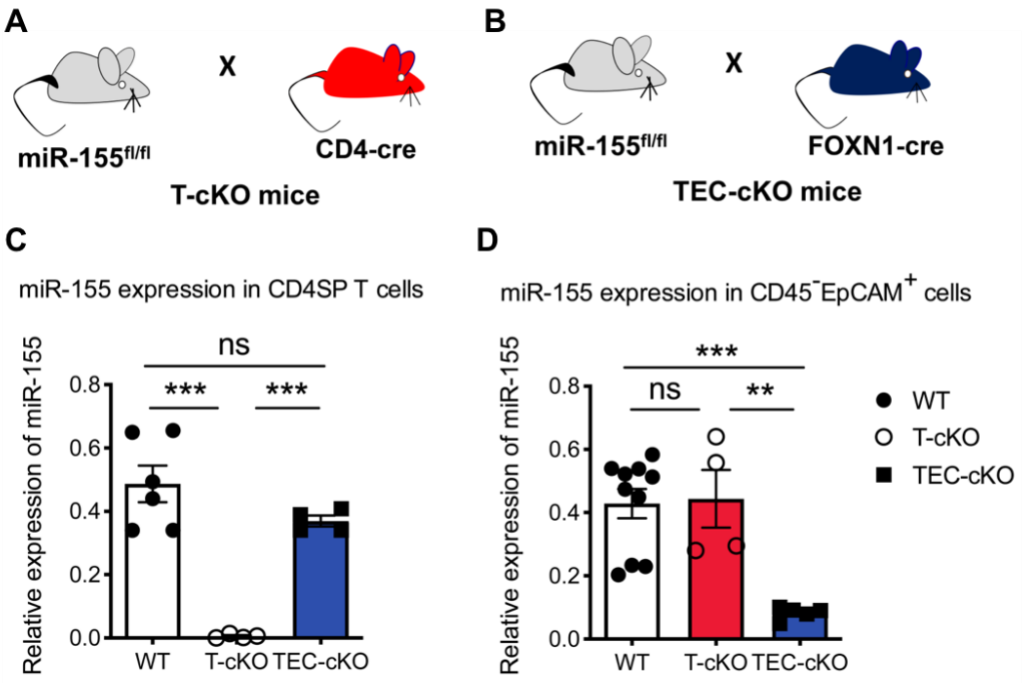

Figure 2.6 Generation of T cell- and TEC-specific miR-155 conditional knockout mice. Schematic of the generation of **(A)** T cell-specific miR-155 conditional knockout mice (T-cKO) and **(B)** TEC-specific miR-155 conditional knockout (TEC-cKO). qPCR analyses of the expression of miR-155 in **(C)** CD4SP T cells ($CD4^+CD8^-$) and **(D)** TECs ($CD45^-EpCAM^+$) isolated from the thymus of 5-6 weeks old WT, T-cKO and TEC-cKO mice. Each symbol represents an individual mouse, and the bar represents the mean. Data are pooled from at least three independent experiments. **, $P < 0.01$; ***, $P < 0.001$.

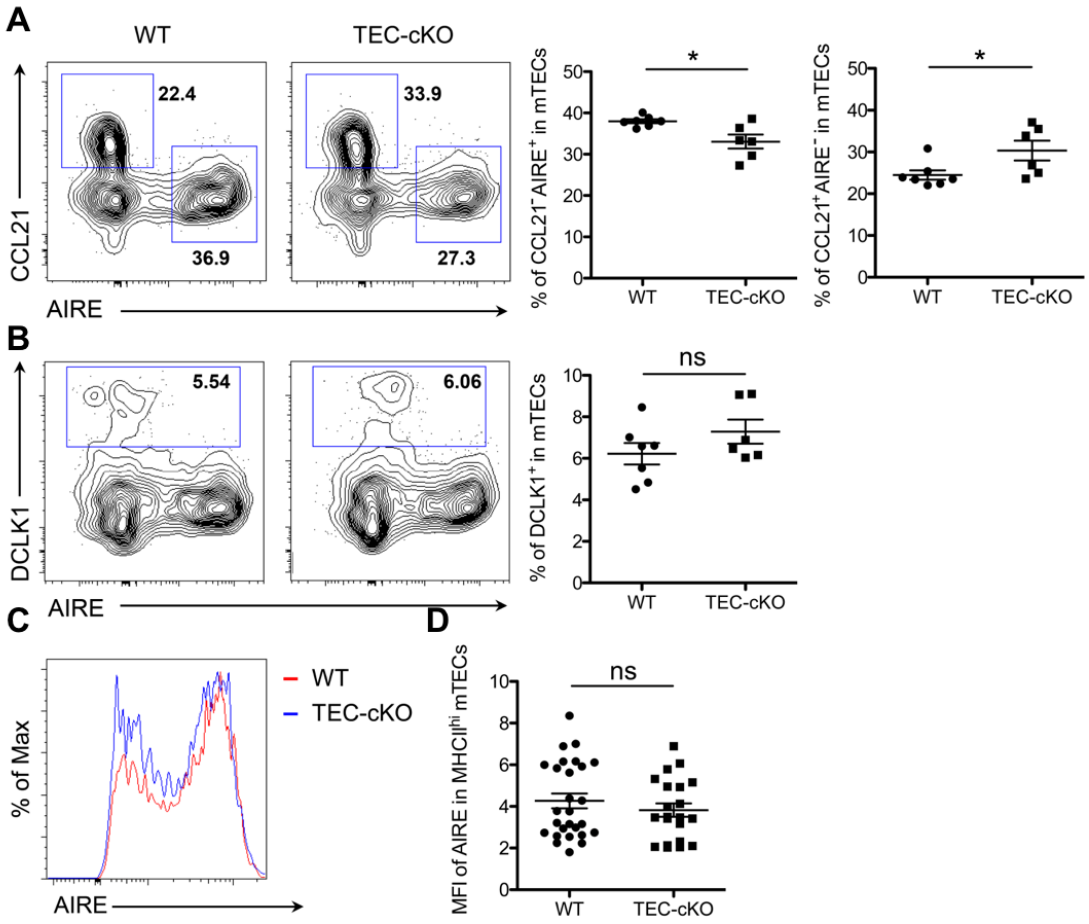

Figure 2.7 Effects of miR-155 deficiency on different mTEC subsets. FACS analysis and frequencies of **(A)** CCL21^{-}AIRE^{+} and CCL21^{+}AIRE^{-} mTECs as well as **(B)** DCLK1^{+} thymic Tuft cells from 5-6 weeks old WT and TEC-cKO mice. **(C)** FACS analyses and **(D)** mean fluorescence intensity (MFI) of AIRE in MHCII^{hi} mature mTECs of TEC-cKO mice compared to WT controls. Each symbol represents an individual mouse, and the bar represents the mean. Data are pooled from at least three independent experiments. *, $P < 0.05$.

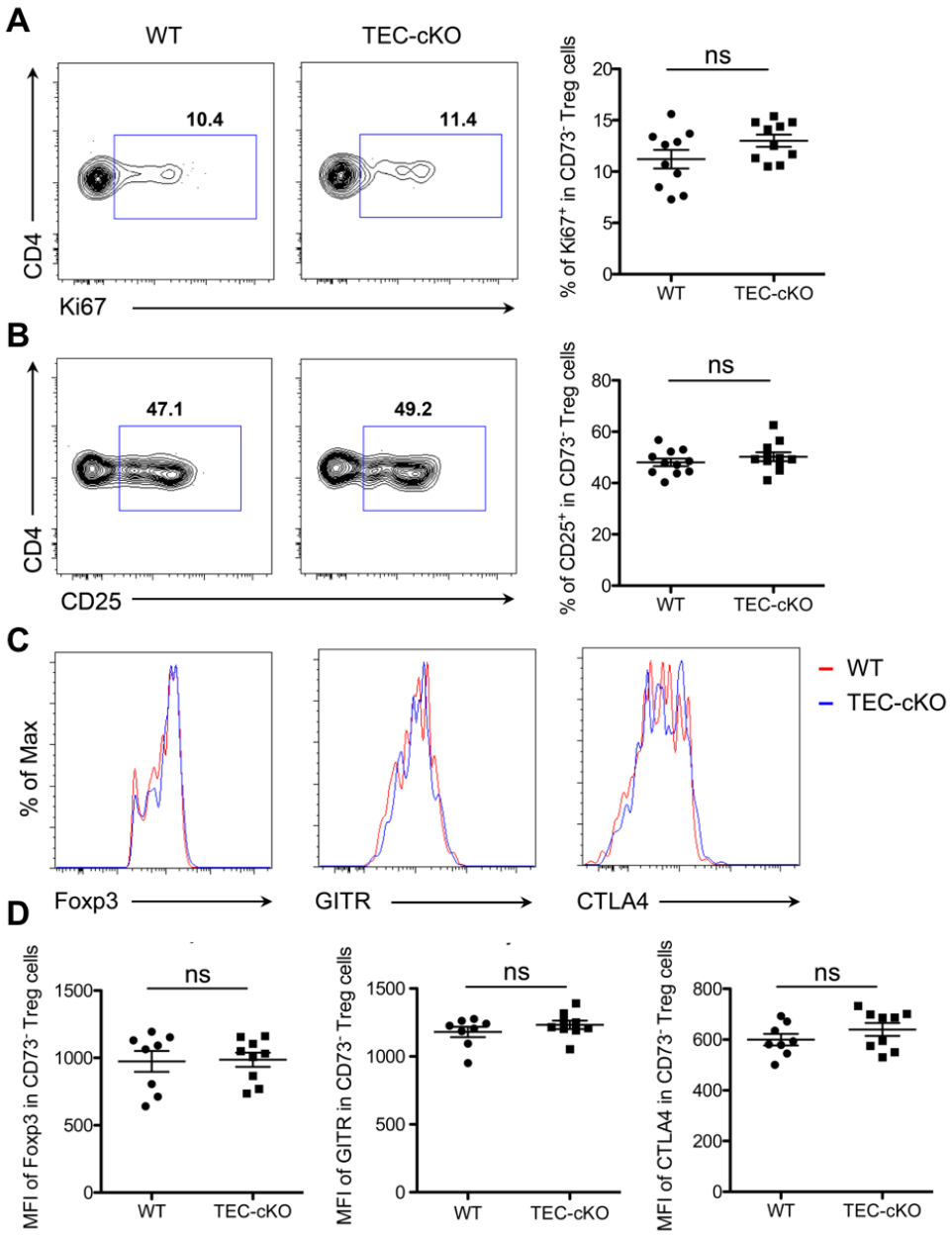

Figure 2.8 Despite reduced frequencies, thymic Treg cells developed in TEC-cKO mice exhibited normal phenotype. FACS analysis and frequencies of **(A)** Ki67^{+} and **(B)** CD25^{+} in thymic CD73^{-} Foxp3^{+} Treg cells from 5-6 weeks old WT and TEC-cKO mice. **(C)** FACS analyses and **(D)** mean fluorescence intensity (MFI) of Foxp3, GITR and CLTA4 in thymic CD73^{-} Foxp3^{+} Treg cells from 5-6 weeks old WT and TEC-cKO mice. Each symbol represents an individual mouse, and the bar represents the mean. Data are pooled from at least three independent experiments.

CHAPTER III: MIR-15/16 CLUSTERS RESTRICT TREG CELL-MEDIATED IMMUNE REGULATION THROUGH LIMITING EFFECTOR TREG CELL DIFFERENTIATION AND FUNCTION

3.1 Introduction

MiRNAs are a class of short non-coding RNAs that mediate gene expression at post-transcriptional regulation (Bartel, 2004). In Treg cells, miRNAs play key roles in mediating Treg development, homeostasis, and function. Ablation of Dicer or Drosha, two key miRNA processing enzymes, in Treg cells leads to severe and even fatal autoimmunity in mice (Liston et al., 2008; Zhou et al., 2008). Consistent with these findings, the roles of individual miRNAs in regulating Treg cell development and functions were gradually uncovered. However, given that an intricate miRNA network integrates multiple pathways in Treg cell biology, the complexity and the severity of the disease phenotypes observed in mice with Treg cell-specific inactivation of the entire miRNA pathway cannot be attributed to the loss of several reported individual miRNAs, thus additional miRNAs essential for controlling other features of Treg cell biology require to be further characterized and elucidated.

Previously miR-15b, a member of the miR-15/16 family, was found to be highly up-regulated in nTreg cells (Kuchen et al., 2010). The miR-15/16 family contains two paralogs: the miR-15a/16-1 cluster on chromosome (chr) 14 (chr 13 in humans) and the miR-15b/16-2 cluster on chr 3 in both mice and humans. Deletion of the miR-15a/16-1 cluster is largely associated with human chronic lymphocytic leukemia (CLL) (Calin et al., 2002). It was shown that the miR-15/16 family restricts B cell proliferation by targeting many genes associated with cell cycle and survival, including Bcl2 (Cimmino et al., 2005; Klein et al., 2010). In T cells, Gagnon et al demonstrated that the miR-15/16 family restrains CD8 memory T cell differentiation, cell cycle, and survival

(Gagnon et al., 2019). In Treg cells, the miR-15/16 family has been implicated in Treg/Th17 cell balance and Treg cell induction (Singh et al., 2015; Wu et al., 2016), suggesting the functional role of the miR-15/16 family in regulating Treg cell biology. However, these studies were mainly conducted in vitro and the physiological roles of the miR-15/16 family in Treg cells remain to be further explored.

In this study, by employing cell-type specific targeting approach, we investigated the function of the miR-15/16 family in Treg cells. We demonstrated that the miR-15/16 family is crucial for restricting eTreg cell differentiation and function. To this end, Treg cell-specific deletion of the entire miR-15/16 family leads to the accumulation of Treg cells in the periphery without significantly impacting their development in the thymus. The elevated Treg cell population observed in mice with Treg cell-specific ablation of the miR-15/16 family can be mostly attributed to a marked increase of $CD25^{lo}Foxp3^{+}$ Treg cells that express high level of ICOS, a phenotype that was found in the eTreg cell population with enhanced immunosuppressive functions (Smigiel, 2014 #174)(Chen, 2018 #28). Moreover, mice harboring miR-15/16 deficient Treg cells developed delayed and attenuated neuroinflammation upon EAE induction, with reduced infiltrating pathological CD4 Tconv cells. Mechanistically, we showed that the miR-15/16 family targets IRF4, a transcriptional factor that is known for its critical role in eTreg cell differentiation and function. Together, our data identifies the miR-15/16 family as negative regulators of Treg cells and demonstrates the importance of miR-15/16 to limit eTreg cells-mediated immune regulation and immune homeostasis.

3.2 Results

3.2.1 The miR-15b/16-2 cluster is predominantly expressed in Treg cells, and is induced by IL-2 along with Foxp3 expression.

A previous miRNA profiling study across different lymphocytes demonstrated that compared to other immune cell subsets, miR-15b and miR-16 are predominantly expressed in Treg cells isolated from the periphery (Kuchen et al., 2010). Consistent with this study, we also detected higher levels of miR-15b cluster members (and miR-15a to a lesser degree) in Treg cells isolated from the spleen compared with their Tconv cell counterpart (Fig. 3.1A). Moreover, elevated levels of the miR-15b cluster, but not miR-15a, could already be detected in thymic Foxp3-expressing Treg cells (Fig. 3.1B). We had previously shown that the differential expression of some miRNAs, for example, miR-27, is tightly regulated in Treg cells and are initiated prior to Foxp3 expression to ensure normal Treg cell development in the thymus (Cruz et al., 2017). To determine whether the miR-15b cluster is induced prior to Treg commitment, we examined the expression of the miR-15b cluster in different thymocyte subsets during T cell development. As shown in Fig. 3.1C, while the expression level of miR-15b remains similarly low in CD4SP Tconv ($CD25^{-}Foxp3^{-}$) cells and the CD4SP $CD25^{hi}Foxp3^{-}$ population, which contains the majority of "Treg precursor cells" (Lio, 2008 #113), it is increased in $Foxp3^{+}$ Treg cells. This result suggests that the miR-15b cluster is induced after Treg cell commitment.

Previous studies showed that several miRNAs that are prevalently present in Treg cells are up-regulated in a Foxp3-dependent manner (Huang et al., 2009; Liu et al., 2015; Lu et al., 2009; Zhang et al., 2021). To examine whether elevated expression of the miR-15b cluster in thymic Treg cells is dependent on Foxp3, we isolated Treg cells (TR) and Treg "wannabe" cells expressing

a Foxp3 reporter null allele (TFN) from healthy heterozygous *Foxp3*$^{GFP/WT}$ and *Foxp3*$^{GPF\text{-}null/WT}$ females (Gavin et al., 2007), respectively. We detected comparable expressions of miR-15b in Treg and Treg "wannabe" cells (Fig. 3.1D), indicating that miR-15b cluster is upregulated in thymic Treg cells in a Foxp3-independent manner.

It has been previously shown that IL-2 signals are transmitted via STAT5 to subsequently induce Foxp3 expression in CD25^{hi}Foxp3^{-} Treg precursor cells (Burchill et al., 2007; Lio & Hsieh, 2008). Since we observed an increased level of miR-15b in Foxp3-expressing Treg cells compared with Treg precursor cells, we next sought to determine whether IL-2 could also drive miR-15b expression in Treg precursor cells. In line with the early work (Lio & Hsieh, 2008), we noted an induction of Foxp3 in CD25^{hi}Foxp3 Treg precursor cells upon IL-2 stimulation *in vitro* (Fig. 3.1E). Moreover, along with up-regulation of Foxp3, increased levels of miR-15b and miR-16 could also be detected, while the level of miR-15a remained unchanged (Fig. 3.1F and G). Collectively, these results suggest that up-regulation of the miR-15b cluster in thymic Treg cells is accompanied by Foxp3 induction in response to IL-2 stimulation, and is further sustained in peripheral Treg cells.

3.2.2 Deletion of miR-15b cluster led to upregulation of miR-15a.

To investigate the role of the miR-15b cluster in Treg cells, we generated mice with Treg-specific ablation of miR-15b cluster by crossing miR-15b/miR-16-2 floxed mice (miR-15b/16-2 ^{fl}) to *Foxp3*^{YFPCre} mice (Treg-bKO). As shown in Fig. 3.2A, we noticed a modest increase in thymic Treg cell frequencies upon Treg cell-specific miR-15b/miR-16-2 ablation. Similarly, a slightly increased Treg cell frequency, albeit not statistically significant, was also observed in the periphery. These modest phenotypes could in part be attributed to the presence of the aforementioned miR-15a cluster. Prior work showed that T cells devoid of either one of these

clusters independently retained high levels of miR-16 expression (Gagnon et al., 2019). Aligned with this observation, we also detected considerable levels of miR-16 expression in Treg cells with either miR-15a or miR-15b cluster depleted. Moreover, miR-15a was also found to be further up-regulated in Treg cells upon miR-15b deletion (Fig. 3.2B). Together, these results suggest that the miR-15a cluster could likely partially compensates for the loss of the miR-15b cluster in Treg cells, and removal of both miR-15/16 clusters is required to understand the role of the miR-15/16 family in Treg cells.

3.2.3 The miR-15/16 family regulates Treg cell homeostasis after Treg cell commitment.

To this end, we generated mice with Treg-specific knockout of both clusters (Treg-DKO) and confirmed the abrogation of miR-15 and miR-16 expression. As shown in Fig. 3.3A, Treg-DKO mice displayed a marked increase in the proportion and absolute numbers of $Foxp3^+$ Treg cells, both in the thymus and in the periphery. As miR-15/16 clusters were deleted only in Foxp3-expressing cells, the observed accumulation of Treg cells could only occur after Treg cell development. Nevertheless, even though elevated expression of the miR-15b cluster was only found after Treg cell commitment, to further determine the role of the miR-15/16 family in Treg cell development, we next generated mice with T cell-specific knockout of both miR-15/16 clusters (T-DKO) (Fig. 3.3B). As shown in Fig. 3.3C, deletion of the miR-15/16 family in T cells led to an increase of thymic Treg cells similar to what was found in Treg-DKO mice, highlighting the importance of miR-15/16 in regulating Treg cell homeostasis, rather than its development. Further supporting the notion, compared with the mature recirculating $CD73^+$ Treg cells, no difference in the frequencies of $CD73^-$ "newly generated" thymic Treg cells could be detected between T-DKO mice and their WT littermates (Fig. 3.3D). Taken together, our results

demonstrate that the elevated expression of the miR-15/16 family in Treg cells acts to control Treg cell homeostasis in the periphery after their development in the thymus.

3.2.4 Loss of the miR-15/16 family led to enhanced eTreg cell differentiation.

Next, we sought to further characterize the impact of miR-15/16 ablation on Treg cell homeostasis. As shown in Fig. 3.4A, miR-15/16-depleted Treg cells exhibited elevated proliferation, which could at least partly account for increased frequencies and absolute numbers of Treg cells found in the Treg-DKO mice. Interestingly, compared with WT littermates, Treg-DKO mice harbored a significant increased $CD25^{lo}Foxp3^{+}$ Treg cell population whereas the frequencies of $CD25^{hi}Foxp3^{+}$ Treg cells remained comparable (Fig. 3.4B). $CD25^{lo}Foxp3^{+}$ cells were previously shown to represent a subset of eTreg cells that depend on continued ICOS signaling for their maintenance particularly at the non-lymphoid organs (Smigiel et al., 2014). Indeed, peripheral $CD25^{lo}Foxp3^{+}$ Treg cells expressed higher levels of ICOS (Fig. 3.4C). In line with these observations, percentages of $ICOS^{+}$ Treg cells, which represent eTreg cells that are associated with enhanced immuno-regulatory functions, are also markedly higher in Treg-DKO mice (Fig. 3.4D). Further characterization of $CD25^{hi}$ and $CD25^{lo}$ Foxp3-expressing Treg cells showed a drastic increase in the percentages of $ICOS^{+}$ population accompanied with enhanced ICOS expression in both Treg cell subsets in mice with Treg cell-specific ablation of the miR-15/16 family (Fig. 3.4E). It should be noted however, that despite the increased frequencies of $CD25^{lo}$ and $ICOS^{hi}$ Treg cells detected in Treg-DKO mice, expression levels of Foxp3 and other known Treg cell-associated molecules were unaffected in the absence of the miR-15/16 family (Fig. 3.4F).

3.2.5 miR-15/16-deficient Treg cells exhibit superior immunosuppressive function and provide enhanced protection against autoimmunity.

Thus far, our data shows that Treg cells devoid of the miR-15/16 family acquire eTreg cell phenotype with elevated ICOS expression, possibly endowing them with enhanced suppressor function (Chen et al., 2018). To test this hypothesis, FACS-sorted miR-15/16-sufficient and -deficient Treg cells were tested for their capacity to suppress the proliferation of Tconv cells in vitro. Indeed, significantly enhanced suppressive activity of Treg cells isolated from Treg-DKO mice was observed across a spectrum of Treg:Tconv cell ratios compared with their WT counterparts (Fig. 3.5A).

To further investigate the physiological relevance of the depletion of the miR-15/16 family on Treg cell function in vivo, we employed a model of experimental autoimmune encephalomyelitis (EAE), a central nervous system (CNS) autoimmune disorder in which autoreactive Th1 cells and Th17 cells have both been suggested to serve as central mediators in promoting disease pathogenesis through the production of proinflammatory cytokines, IFNγ, IL-17 and GM-CSF, respectively (Codarri et al., 2011; El-Behi et al., 2011; Ponomarev et al., 2007; Stromnes et al., 2008). As shown in Fig. 3.5B, mice harboring miR-15/16-deficient Treg cells exhibited delayed onset of EAE along with decreased disease severity, as compared to WT controls. Consistently, accompanied with increased $CD25^{lo}$ and $ICOS^{hi}$ Treg cell populations, we also observed a significant decrease of infiltrating $CD4^{+}$ T cells in the CNS that produce IFNγ and GM-CSF (as well as IL-17 albeit not statistically significant) in Treg-DKO mice even at an early phase of the disease (Day 15; Fig. 3.5C).

3.2.6 The miR-15/16 family directly targets Irf4 and negatively regulates the eTreg cell program.

In order to shed light on the mechanistic targets of the miR-15/16 family that control eTreg differentiation and function, we took advantage of previous results obtained from high-throughput sequencing of RNA isolated by cross-linking immunoprecipitation (HITS-CLIP) generated from in vitro activated CD4+ T cells, which globally map AGO-miRNA complex binding to target RNAs that inform the identification of functional miRNA-mRNA interactions in CD4 T cells (Loeb et al., 2012). Through searching miR-15 seed matches within the HITS-CLIP-identified AGO-bound regions, we identified IRF4, a transcription factor that has been previously implicated in instructing eTreg cell differentiation (Alvisi et al., 2020; Cretney et al., 2011), as a potential miR-15b target (Fig. 3.6A). Supporting this notion, our luciferase reporter studies confirmed that miR-15b can directly repress IRF4 (Fig. 3.6B). Furthermore, Treg cells isolated from Treg-DKO mice express higher amounts of IRF4 at both mRNA and protein levels (Fig. 3.6C and D). Therefore, our data suggest that miR-15 could restrict eTreg cell differentiation and function at least in part through targeting IRF4.

To confirm this hypothesis and to gain further molecular insights into the mechanistic aspect of how miR-15/16 controls eTreg cell differentiation and function, we isolated miR-15/16-sufficient and -deficient Treg cells from healthy heterozygous *Foxp3*$^{YFPCre/+}$ female WT and Treg-DKO mice and performed bulk RNA sequencing (RNA-seq) analysis. Transcriptomic analysis revealed a significant amount of differentially expressed genes are enriched in Gene Ontology terms, including T cell activation, nuclear division, and negative regulation of immune system process, reflecting pronounced changes in the subset of genes associated with the cell cycle and immune regulation (Fig. 3.7A). Interestingly, in accordance with our observations of a substantial

increase of IRF4 expression in Treg cells devoid of the miR-15/16 family, many previously reported IRF4-dependent genes (Cretney et al., 2011; Zheng et al., 2009), including *Il10, Cd28, Stx11, Fgl2, Alcam, Ctla2a, Cd200r1, Itgb1* and *Mmd,* were up-regulated in miR-15/16-deficient Treg cells (Fig. 3.7B). These differentially expressed genes are largely overlapped with the previously reported eTreg cell gene signatures (Dias et al., 2017). In addition to IRF4-dependent genes, several other genes such as *Lag3, Tigit, Gata1*, and *Igfbp4* that are known to be differentially expressed in eTreg cells were also found in our RNA-seq analysis of miR-15/16-deficient Treg cells (Fig. 3.7B), suggesting that the miR-15/16 family could also control eTreg cell differentiation and function through an IRF4-independent manner. Of note, among all the IRF4-dependent eTreg cell differentially expressed genes, *Nrn1*, a transcript of neurotrophic factor Neuritin 1, is significantly upregulated in miR-15/16-deficient Treg cells. Neuritin 1 has been previously implicated in promoting Treg cell expansion and balancing "central"- and "effector"-like Treg populations and function (Lucas & Lucas, 2021). It has also been suggested to be a key suppressor molecule that follicular Treg (Tfr) cells produce to control plasma cell responses during germinal center (GC) reactions (Gonzalez-Figueroa et al., 2021). Consistent with the RNA-seq results, elevated expression of Nrn1 expression level was detected in miR-15/16-deficient Treg cells in our qPCR analysis (Fig. 3.7C). A further survey of *Nrn1* 3'UTR reveals a putative miR-15 binding site, suggesting that the miR-15/16 family could also directly target Nrn1 to regulate Treg cell effector function (Fig. 3.7D). Collectively, our results demonstrate that through targeting a network of genes from transcription factor IRF4 to effector molecule Nrn1 that are crucial for eTreg differentiation and function, the miR-15/16 family emerges as key regulators in controlling Treg cell biology, particularly via maintaining the optimal balance between central and effector Treg cell populations.

3.3 Discussion

Accumulating evidence suggests that Treg cells are heterogeneous and made up of different subsets with distinct phenotypical and functional characteristics (Shevyrev & Tereshchenko, 2019). The diversity of Treg cells is driven by specialized programs that are regulated at both the transcriptional and post-transcriptional levels.

In this study, we demonstrated that the miR-15/16 family controls eTreg differentiation and function at least in part through targeting IRF4. To this end, transcriptional analysis revealed many genes that were differentially expressed in miR-15/16-deficient Treg cells overlapped with previously reported IRF4-dependent genes as well as eTreg cell gene signature (Dias et al., 2017; Zheng et al., 2009). Nevertheless, many eTreg genes differentially expressed in Treg cells devoid of the miR-15/16 family are IRF4-independent, suggesting that the phenotypic and functional changes in miR-15/16-deficient Treg cells cannot be solely attributed to the lack of miR-15/16-mediated suppression of IRF4. Moreover, even in those IRF4-dependent genes, the miR-15/16 family can also directly target them to ensure proper regulation. Indeed, we also identified that *Nrn1*, whose expression was previously shown to be driven by IRF4 (Zheng et al., 2009), harbors a putative miR-15/16 binding site at its 3'UTR and can be directly inhibited by the miR-15/16 family. Besides *Irf4* and *Nrn1*, it is likely that the miR-15/16 family targets a network of genes that are involved in eTreg differentiation and function. Integrative analysis of HITS-CLIP and RNA-seq data will help shed light on identifying additional targets that are pivotal in this process.

eTreg cells are usually characterized by upregulated expression of effector molecules, such as CTLA4, ICOS, KLRG1, and TIGIT (Koizumi & Ishikawa, 2019; Shevyrev & Tereshchenko, 2019). Interestingly, despite our consistent observation of increased expression of several molecules, such as ICOS, TIGIT and GITR in miR-15/16-deficient Treg cells, we didn't find a

noticeable difference in CTLA4 and KLRG1 expression. This suggests the presence of a diversity of distinct eTreg subsets that are phenotypically and functionally different. Future investigation of different subsets of eTreg cells is critical to help us better understand their distinct functions in different disease settings. In support of this notion, a previous work by Yang et al. showed that eTreg cells heterogeneously express TCF1 and LEF1. $TCF^{-}LEF^{-}$ eTreg cells express high levels of eTreg-related molecules such as *Cd44*, *Icos*, *Tigit*, *Irf4*, and *Prdm1* while $TCF^{+}LEF^{+}$ eTreg cells can further differentiate into the Tfr cell lineage with the expression of Tfr cell signature genes such as *Bcl6* and *Cxcr5* (Yang et al., 2019). Interestingly, we did observe an increase of *Cxcr5* transcript levels in miR-15/16-deficient Treg cells from our RNA-seq results. Moving forward, studies using single cell RNA sequencing approach could help address concerns about the heterogeneity of eTreg cells and further unravels the role of this miRNA family in establishing Treg cell identity of different fates.

3.4 Materials and Methods

Mice

miR-15a/16-1^{fl}, miR-15b/16-2^{fl}, and miR-15a/16-1^{fl};miR-15b/16-2^{fl} mice were bred with $Foxp3^{YFPCre}$ mice, respectively, to obtain mice with Treg cell-specific deletion of miR-15a/16-1(Treg-aKO), miR-15b/16-2 (Treg-bKO) and both miR-15/16 clusters (Treg-DKO). miR-15a/16-1^{fl};miR-15b/16-2^{fl} mice were bred with $dLCK^{Cre}$ mice to obtain T cell-specific deletion of both miR-15/16 clusters (T-DKO). All mice were maintained and handled in accordance with the Institutional Animal Care and Use Guidelines of UCSD and National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and the ARRIVE Guidelines.

Flow cytometry and sorting

For T cell analysis, single-cell suspensions of thymus, spleen, lymph nodes, as well as as well as lymphocyte isolation in the brain and spinal cord were prepared as described previously. Cells were first stained with Ghost Dye Red 780 (Tonbo Biosciences) followed by surface antibody staining for CD4 (RM4-5), CD8a (53-6.7), CD44 (IM7), CD62L (MEL-14), CD25 (PC61), GITR (DTA-1), ICOS (7E.17G9, C398.4A), CD73(TY/11.8), Thy1.1(HIS51). Intracellular staining for Foxp3 (FJK-16s), Ki67 (SolA15), CTLA-4 (UC10-4B9), YFP/GFP(FM264G), was completed after fixation and permeabilization with Foxp3/Transcription Factor Staining Kit according to manufacturer protocol (Tonbo). To detect IFNg (XMG1.2), IL-17 (eBio17B7) and GM-CSF (MP1-22E9) cytokine production, cells were stimulated in a 96-well plate with 50 ng/ml PMA, 0.5 mg/ml ionomycin, and 1 mg/ml Brefeldin A (all from Sigma-Aldrich) in complete 5% RPMI media for 4 hours at 37C before staining. For thymic Treg cell analysis, total tTreg cells were gated on $Foxp3^+$ cells from the $CD4^+CD8^-$ population. For nascent and recirculating tTreg cells, $CD73^-$ and $CD73^+$ cells were first gated from the $CD4^+CD8^-$ population, followed by $Foxp3^+$ gating. Cells were fixed in 2 % paraformaldehyde prior to analysis by flow cytometry. FACS Data were collected by BD LSRFortessa or BD LSRFortessa X-20 (BD Biosciences) and analyzed by FlowJo (Tree star). Finally, BD BD FACSAria Fusion (BD Biosciences) was used for cell sorting.

Quantitative PCR analysis

For quantification of the expression of miR-15/16 in different thymocyte subsets, DN, DP, CD8SP, $CD25^-Foxp3^-$CD4SP, $CD25^+Foxp3^-$CD4SP and $Foxp3^+$CD4SP from $Foxp3^{YFP}$ reporter mice were sorted on BD FACSAria II (BD Biosciences) with a purity of >95%, followed by RNA isolation using miRNeasy kit (Qiagen) according to the manufacturer's instructions. To determine

expression of miR-15a, miR-15b and miR-16, TaqMan MicroRNA Assay (Thermo Fisher Scientific) was performed. For other gene detection, cDNA was generated using iScript cDNA Synthesis Kit (Bio-Rad), followed by qPCR reactions using SYBR Green PCR Mix (Thermo Fisher Scientific). The primer sequences used were as follows: *Irf4*: 5'-GAACGAGGAGAAGAGCGTCTTC-3' (F), 5'-GTAGGAGGATCTGGCTTGTCGA-3' (R); *Nrn1*: 5'-TGTTTGCTCAAGCTGGGCGACA-3' (F), 5'-CTTCCTGGCAATCCGTAAGAGC-3' (R); *Foxp3*: 5'-GCGAAAGTGGCAGAGAGGTA-3' (F), 5'-TCCAAGTCTCGTCTGAAGGC-3' (R); *Il10*: 5'-ATCGATTTCTCCCCTGTGAA-3' (F), 5'-TGTCAAATTCATTCATGGCCT-3' (R); *Icos*: 5'-TGACCCACCTCCTTTTCAAG-3' (F), 5'-TTAGGGTCATGCACACTGGA-3' (R); *Gapdh*: 5'-CGTCCCGTAGACAAAATGGT -3' (F), 5'-TCAATGAAGGGGTCGTTGAT-3' (R).

In vitro IL-2 Stimulation

$CD25^{hi}YFP^{-}$ Treg precursor cells from $Foxp3^{YFPCre}$ reporter mice were sorted on a FACSAria Fusion (BD Biosciences) into complete RPMI, followed by stimulation with 500 unit/ml IL-2 for 24 hrs at 37 °C.

In vitro suppression assay

For in vitro suppression assays, Treg cells (T_R) and 'effector' T cells (T_E) were purified by negative selection using CD4-specific MACS kit (Biolegend), followed by sorting on a FACSAria cell sorter (Becton Dickinson). Antigen-presenting cells were prepared from wild-type B6 splenocytes by T-cell depletion. Effector T cells (5×10^4 cells $well^{-1}$) were co-cultured with Treg cells at indicated ratios in the presence of mytomycin C-treated antigen-presenting cells

(1.5×10^5 cells $well^{-1}$) in 96-well plates in complete RPMI1640 medium supplemented with 10% FBS and CD3 antibody (1 μg ml^{-1}) for 72 h.

Luciferase reporter assay

The 3'UTR sequences of *Irf4* were amplified from mouse genomic DNA and cloned into pSiCheck2 vector (Promega). Site-directed mutagenesis (Agilent) was performed to obtain mutants of *Irf4* 3'UTR. Indicated 3'UTR WT or mutant plasmids were transfected into HEK293T (ATCC CRL-3216) cells along with either a miR-15b-expressing plasmid or a control empty vector. Luciferase activity was determined by the Dual luciferase reporter assay system (Promega) according to the manufacturer's instructions at 24 hr after transfection.

Immunostaining

FACS purified Treg cells from $Foxp3^{YFPCre}$ reporter mice were subjected to lysis with RIPA buffer supplemented with 1 mM PMSF for 30 minutes. Cell debris was removed by centrifugation at 14,000 rpm for 15min at 4 °C , and the supernatants were transferred to fresh tubes. Cell lysates were separated by SDS-PAGE followed by a nitrocellulose membrane (Bio-Rad Laboratories) transfer. Antibodies against β-actin (AC-74), IRF4 (E8H3S) were used to visualize the corresponding proteins. Images were acquired on an LSM 700 system (Carl Zeiss Inc.) Quantification of proteins was calculated with ImageJ (National Institutes of Health).

Gene expression profiling

$CD4^+Foxp3^+$ Treg cells were sorted on FACSAria II cell sorter (BD Biosciences) from heterozygous $Foxp3^{YFPCre/+}$ DKO and WT female mice, followed by total RNA isolation using a

miRNeasy Kit (QIAGEN). cDNA was synthesized as previously described. Single-end 50 base-pair RNA sequencing was performed on the Illumina HiSeq 4000. Alignment was performed using STAR (Dobin et al., 2013) against the mouse genome (mm10). Differential expression was tested using DESeq2 (Love et al., 2014) with a cutoff of 1.5 fold change and p-adj 0.05. GO biological processes associated with genes (1.5 fold change, pvalue 0.05) were queried using clusterProfiler (Yu et al., 2012), and the top GO categories with the most significance were selected.

Statistical Analyses

A 2-tailed Student's t test (or 1-way ANOVA for studies with more than two groups) was done on all reported data using Prism software (GraphPad) (*P < 0.05; **P < 0.01; ***P < 0.001; ns: not significant.). All experiments were performed independently at least three times to ensure the reproducibility of the data.

Chapter 3, in part, includes parts of a manuscript in preparation for publication. **Dong, J.**, Huth, W. J., Lin, L. L., Chen, M. C.& Lu, L. F. 2022. MiR-15/16 clusters restrict Treg cell-mediated immune regulation through limiting effector Treg cell differentiation and function. This dissertation author was the first author of the article.

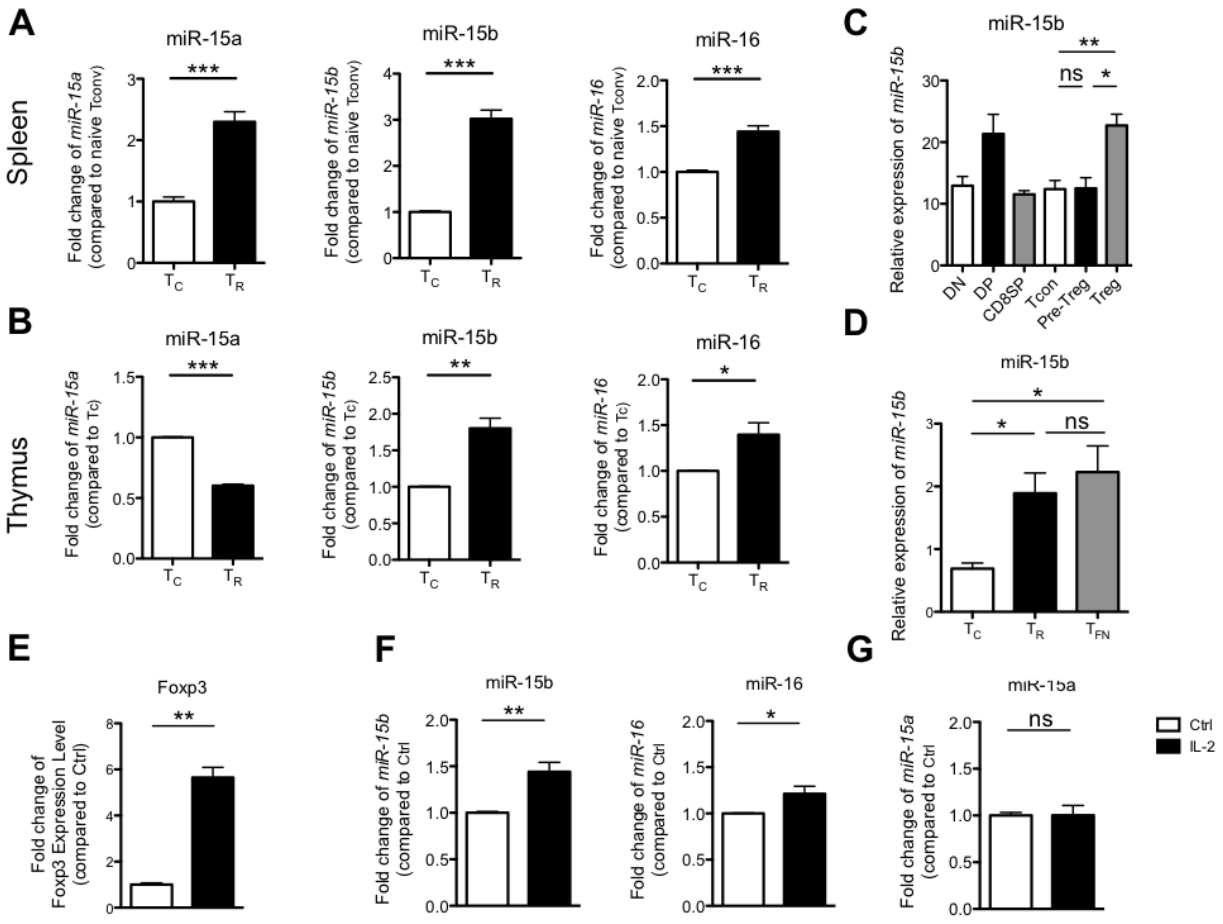

Figure 3.1 The miR-15b/16-2 cluster is predominantly expressed in Treg cells, and is induced by IL-2 along with Foxp3 expression. qPCR analysis of miR-15a, miR-15b, miR-16 expression in $CD4^+CD25^-Foxp3^-$ Tconv (T_C) cells and $CD4^+Foxp3^+$ Treg (T_R) cells isolated from **(A)** spleen and **(B)** thymus. **(C)** qPCR analysis of miR-15b expression in different thymocyte subsets. **(D)** qPCR analysis of miR-15b expression in T_C, T_R and T_{FN} cells isolated from thymus. qPCR analyses of **(E)** Foxp3, **(F)** miR-15b, miR-16 and **(G)** miR-15a expression in sorted Treg precursor ($CD25^{hi}Foxp3^-$) cells stimulated with 100U/ml IL-2 for 24 hours *in vitro*. The n-fold changes on the basis of corresponding control groups were shown. The bar represents the mean. Data are pooled from at least three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

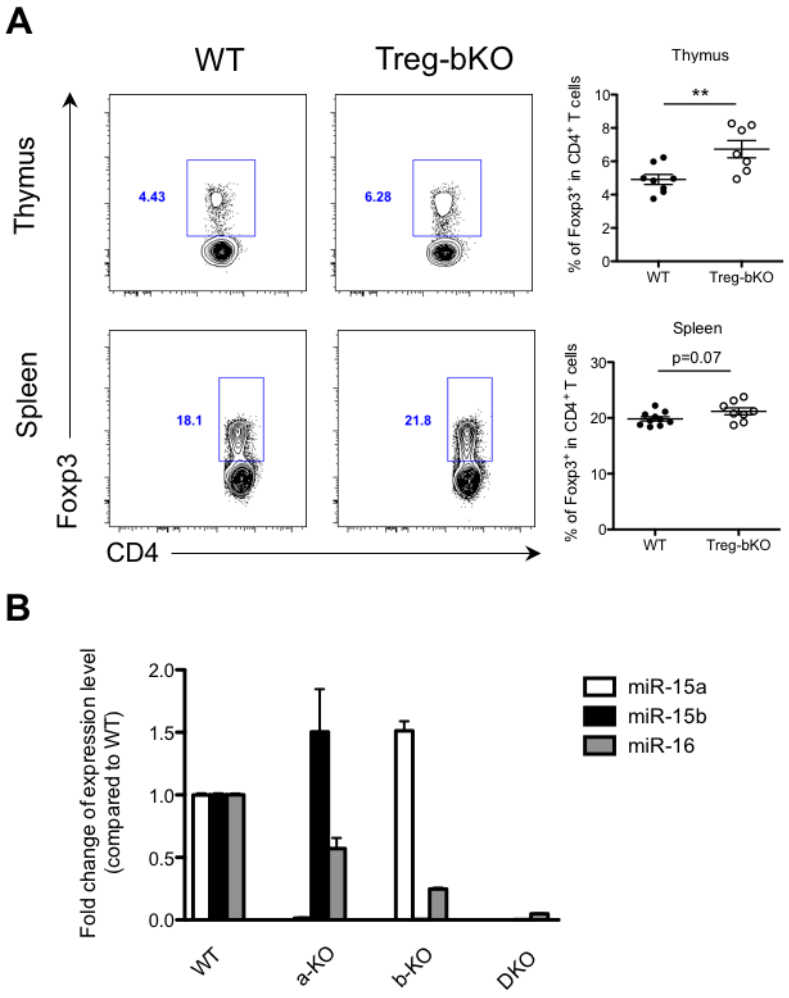

Figure 3.2 Deletion of miR-15b cluster led to upregulation of miR-15a.
(A) FACS analysis and frequencies of Foxp3$^+$ Treg cells in the thymus and spleen from 8-12 weeks old WT and Treg-bKO mice. **(B)** qPCR analyses of miR-15a, miR-15b and miR-16 expression in splenic Treg cells isolated from WT, Treg-aKO and Treg-bKO mice. The n-fold changes on the basis of corresponding control groups were shown. Each symbol represents an individual mouse, and the bar represents the mean. Data are pooled from at least three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

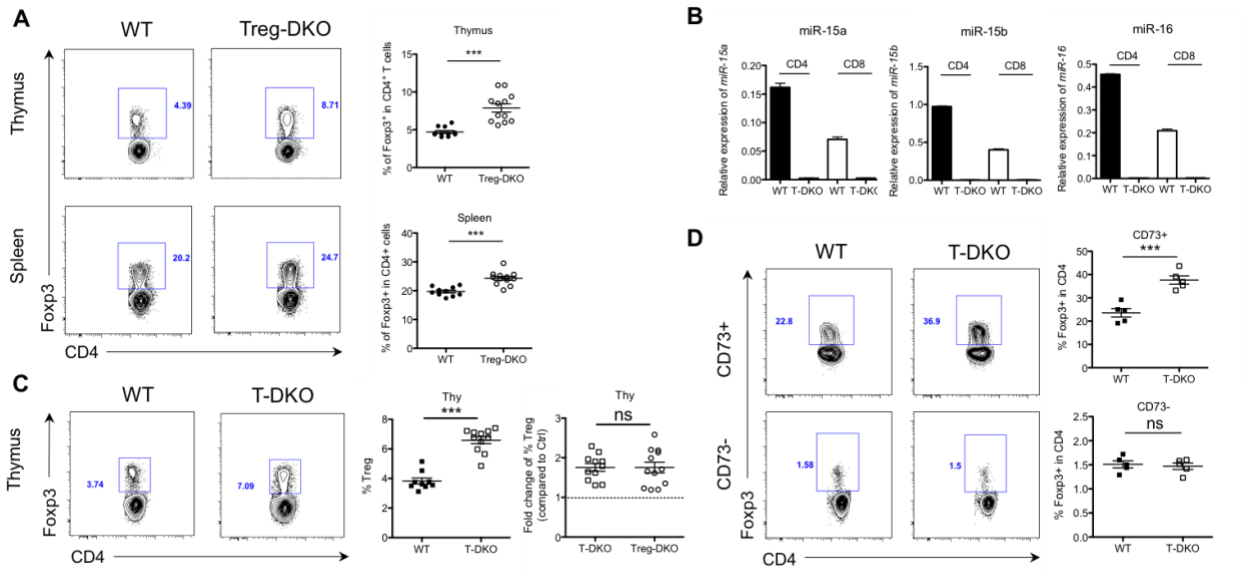

Figure 3.3 The miR-15/16 family regulates Treg cell homeostasis after Treg cell commitment.

(A) FACS analysis and frequencies of Treg cells in the thymus and spleen from 10-16 weeks old WT and Treg-DKO mice. **(B)** qPCR analyses of miR-15a, miR-15b and miR-16 expression in total CD4 and CD8 T cells in the spleen isolated from WT and T-DKO mice. **(C)** FACS analysis, frequencies of total Foxp3$^+$ Treg cells in the thymus of WT and T-DKO mice. Fold change of % of total thymic Treg cells in T-DKO and Treg-DKO mice compared the corresponding WT littermates. **(D)** FACS analysis and frequencies of CD73$^+$ Foxp3$^+$ and CD73$^-$ Foxp3$^+$ Treg cells in the thymus of WT and T-DKO mice. Each symbol represents an individual mouse, and the bar represents the mean. Data are pooled from at least three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

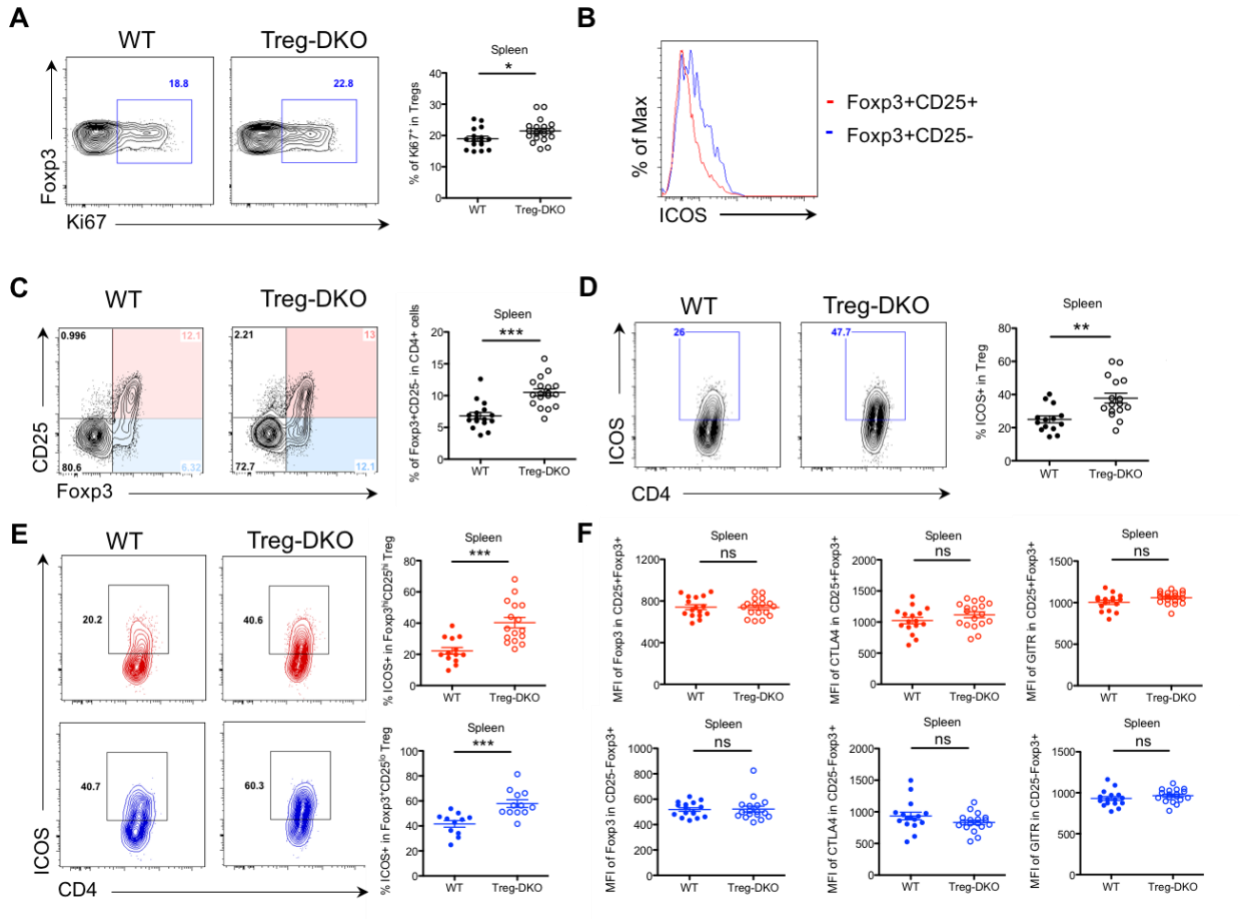

Figure 3.4 Loss of the miR-15/16 family led to enhanced eTreg cell differentiation.
(A) FACS analysis and frequencies of Ki67^{+} in splenic Foxp3^{+} Treg cells from 10-16 weeks old WT and Treg-DKO mice. **(B)** FACS analysis of ICOS expression in CD25^{hi}Foxp3^{+} (red) and CD25^{lo}Foxp3^{+} (blue) Treg cells from WT mice. FACS analysis and frequencies of **(C)** CD25^{lo}Foxp3^{+} cells and **(D)** ICOS^{+}Treg cells in the spleen from WT and Treg-DKO mice. **(E)** FACS analysis and frequencies of ICOS^{+} cells in splenic CD25^{hi} Foxp3^{+} (red) and CD25^{lo} Foxp3^{+} (blue) Treg cells from WT and Treg-DKO mice. **(F)** Mean fluorescence intensity (MFI) of Foxp3, CLTA4 and GITR in splenic CD25^{hi} Foxp3^{+} and CD25^{lo} Foxp3^{+} Treg cells from WT and Treg-DKO mice. Each symbol represents an individual mouse, and the bar represents the mean. Data are pooled from at least three independent experiments.

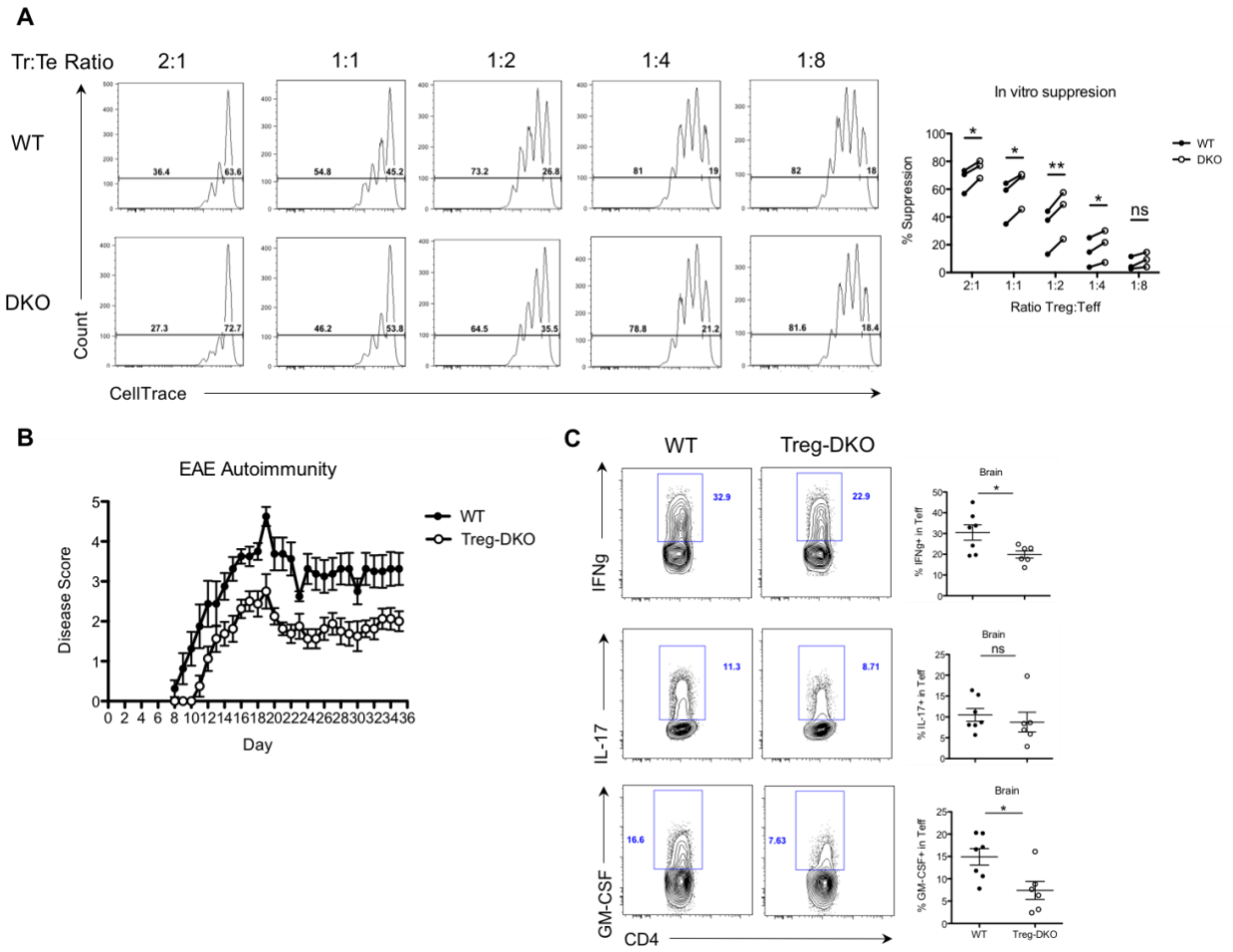

Figure 3.5 miR-15/16-deficient Treg cells exhibit superior immunosuppressive function and provide enhanced protection against autoimmunity.
(A) Treg cells (T_R) isolated from Treg-DKO or WT control littermates were subjected to in vitro suppression analysis at indicated ratios of responder T cells (T_E). Data are representative of three independent experiments. **(B)** Clinical scores of Treg-DKO and WT control mice induced for EAE. The mice were daily monitored and scored for clinical diseases. **(C)** FACS analyses and frequencies of $IFNg^+$, $IL\text{-}17^+$, and $GM\text{-}CSF^+$ cells in $Foxp3^-CD4^+$ effector T cells in CNS from Treg-DKO and WT control littermates on day 15 post induction. Each symbol represents an individual mouse, and the bar represents the mean. Data are pooled from at least three independent experiments.

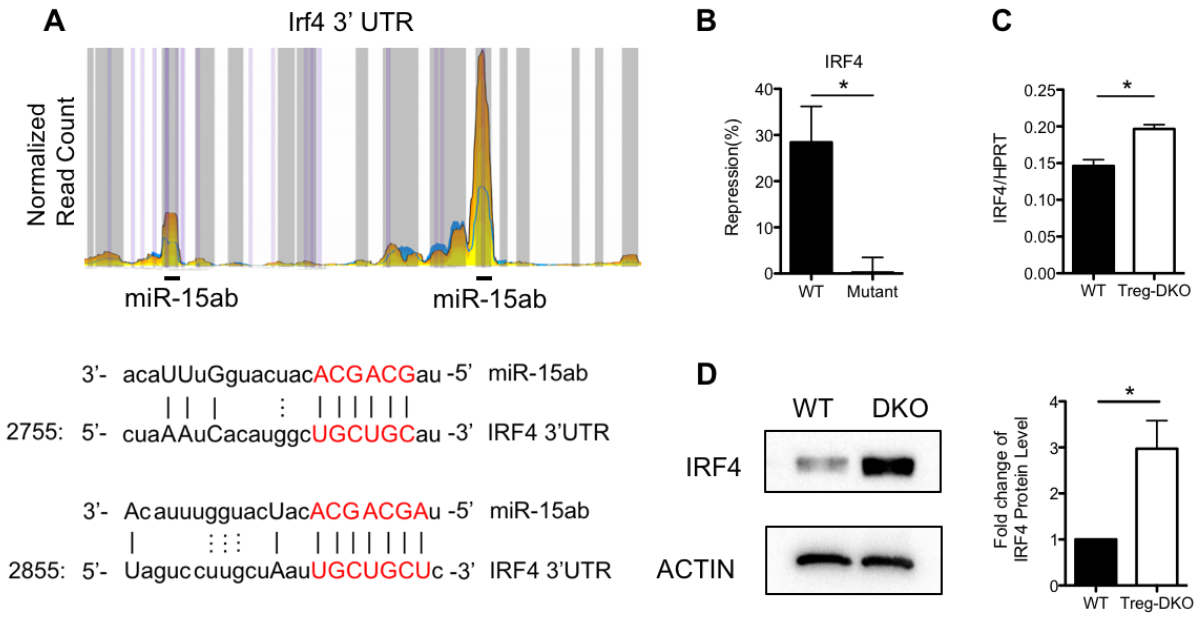

Figure 3.6 The miR-15/16 family directly targets *Irf4*.
(A) HITS-CLIP analysis (the numbers represent the nucleotide position related to the start of the 3' UTR) and sequence alignments of the putative miR-15ab binding sites in 3'UTR of *Irf4*. **(B)** Ratios of repressed luciferase activity of cells with *Irf4* 3' UTR with or without mutations in the seed sequences in the presence of miR-15b compared with cells transfected with empty vector. **(C)** qPCR analysis of the expressions of *Irf4* in sorted Foxp3^{+} Treg cells from 8-12 weeks old WT and Treg-DKO mice. **(D)** Immunoblot analysis of IRF4 expression in Treg cells isolated from spleen. Densitometric IRF4 expression values were normalized to β-actin expression values and n-fold increase on the basis of each corresponding WT. The bar represents the mean. Data are pooled from at least three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

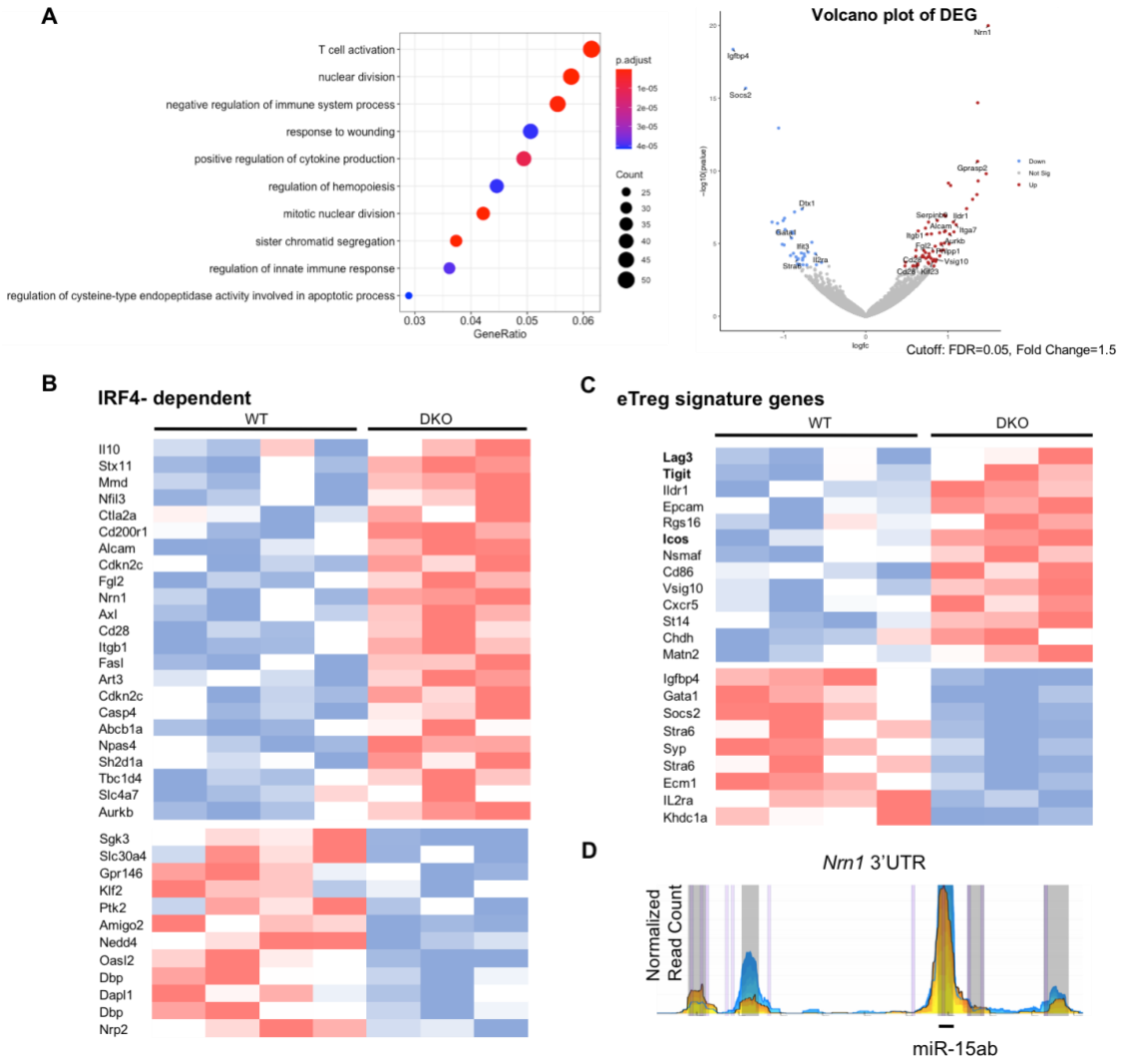

Figure 3.7 The miR-15/16 family negatively regulate a network of genes in eTreg cell program.

(**A**) Annotated GO biological processes (left) were assigned to genes differentially expressed in Treg cells with or without miR-15/16 clusters expression, as determined by RNA-seq. Log_2 fold change (FC) of the DE genes (false-discovery rate < 0.05) between miR-15/16-deficient and -sufficient Treg cells; each dot represents one gene, and the representative genes were annotated and colored in the volcano plot. Heatmap of representative (**B**) IRF4-dependent genes and (**C**) genes associated with eTreg differentiation that were differentially expressed in miR-15/16 clusters-deficient and -sufficient Treg cells. The relative expression of genes is shown and is color coded. **(D)** HITS-CLIP analysis of the putative miR-15ab binding site in 3'UTR of *Nrn1*.

CHAPTER IV: CONCLUDING REMARKS

Over the past two decades, thanks to the technological advancements in miRNA target identification and the establishment of a vast number of genetic tools, our knowledge of the role of miRNAs in regulating Treg biology and T cell immunity has grown tremendously. However, with our understanding of miRNA-mediated control of immune responses gets deeper, new questions have also arisen.

One of the most interesting findings from recent studies is the fact that several miRNAs that serve as important gene modulators in a given T cell population are also highly expressed and play key functions in other cell types that T cells closely interact with (Cho et al., 2018; Dong et al., 2021). For example, in Chapter 2, we demonstrated that miR-155, a miRNA whose expression in T cells is critical for Treg cell development and homeostasis, also plays a pivotal role in driving thymic Treg cell differentiation through the promotion of mTEC maturation. To this end, miR-155 is preferentially expressed in mature $CD80^{hi}MHCII^{hi}$ mTECs in part via a RANK signaling-dependent manner. Elevated expression of miR-155 in mTECs is important for maintaining optimal mTEC maturation and consequently impacts the output of Treg cells generated in the thymus. Mechanistically, we have further demonstrated that miR-155 ensures proper mTEC maturation through targeting a network of molecules (including TGFβR2, SMAD2/3, and RNF111) within the TGFβ signaling pathway. As the TGFβ signaling pathway had been previously shown to play a key role in limiting the maturation and expansion of mTECs(Hauri-Hohl et al., 2014), our work reveals a previously unappreciated role of miR-155 in safeguarding mTEC maturation through counteracting the negative effects from the continuous presence of intrathymic TGFβ, thereby establishing an optimal thymic microenvironment favorable for thymic Treg cells. This study is the first demonstration of an individual miRNA with functional

importance in driving Treg cell differentiation in both T cell-intrinsic and -extrinsic manners. It also suggests that additional attention should be paid to stromal cells residing in the same microenvironments to better understand the biological impact of a given miRNA on a selective T cell response or other immunological processes. Nevertheless, the detected similar expression patterns of miRNAs in T cells and their interacting partners could not be simply explained by the shared environmental signals they receive. These miRNAs do not appear to target the same set of genes in the two interacting populations despite promoting concerted responses (Dong et al., 2021; Lu et al., 2009). Thus, it remains to be further investigated whether these are unique cases or represent a common mechanism by which miRNAs control immune response.

On the other hand, miRNAs can act directly as pivotal regulatory elements to fine-tune gene expression networks in T cells, thus ensuring the generation of optimal T cell responses without causing autoimmunity (Cho et al., 2021; Mehta & Baltimore, 2016). To this end, in Chapter 3, we demonstrated that the miR-15/16 family serves as negative regulators in modulating eTreg cell differentiation and function. While IL-2 signaling is essential for Treg cell development and homeostasis, it also plays a role in driving the elevated expression of the miR-15/16 family to control their development into eTreg cells that exhibit superior immunosuppressive function. Supporting this notion, we observed eTreg cells express an increased level of the miR-15/16 family, suggesting that the presence of the miR-15/16 family likely serves as a negative feedback loop to prevent exaggerated immunosuppression. While the differentiation and expansion of the eTreg cell population can help control autoimmune inflammation, it can also prevent effective immune surveillance. To this end, it has been previously reported that the abundance of $IRF4^+$ and $ICOS^+$ Treg cells correlated with poor prognosis in patients with multiple human cancers (Alvisi

et al., 2020). Consistently, our preliminary tumor studies also showed that mice harboring miR-15/16-deficient Treg cells exhibited compromised anti-tumor immunity.

Despite the increasing appreciation of miRNAs as crucial modulators and oscillators in immunological circuits, many questions remain to systematically understand the temporal and spatial regulations of miRNA expression and their functions in a context-dependent manner. For example, how do individual miRNAs change their expression patterns in response to an immune stimulus or along immune cell differentiation stages? Subsequently, how does it affect their mRNA "targetome" to coordinate downstream gene networks and signaling pathways? Answering these questions requires the combination of extensive knowledge of miRNA functions and integrative technologies, such as RNA-seq and eCLIP-seq for further analysis of miRNA-target interactions. Nevertheless, our understanding of miRNA-dependent regulation of Treg cell biology has greatly helped gain mechanistic insights into Treg cell-mediated immune regulation, which could subsequently lead to the discovery of potential novel therapeutic targets. For example, in Chapter 3, we found one of the top differentially expressed genes in miR-15/16-deficient Treg cells is Nrn1, a gene that encodes Neuritin 1, which is a neuropeptide most widely studied in neurons. The role of Neuritin 1 in the immune system remains poorly characterized. However, a recent study reported that Tfr cells in the germinal center could secrete Neuritin 1, which suppresses the development of B-cell-driven autoimmunity and IgE-mediated allergic responses (Gonzalez-Figueroa et al., 2021). In our study, we found miR-15/16-deficient Treg cells exhibited enhanced proliferation and effector function, providing better protection from autoimmunity (e.g. EAE) in mice. More interestingly, in our preliminary analysis of *Foxp3*$^{YFPCre/+}$ female heterozygous Treg-DKO mice which harbor both miR-15/16-deficient and –sufficient Treg cells at the same time, the miR-15/16-deficient Treg cells seem to promote the expansion and effector

state transition in their surrounding WT Treg cells that reside in the same tissue microenvironment. Given that Neuritin 1 is a soluble factor that can be produced by Treg cells and has been previously implicated to promote eTreg cell differentiation and expansion (Gonzalez-Figueroa et al., 2021; Lucas & Lucas, 2021), it is plausible that miR-15/16-deficient Treg cells promote not only their own expansion and effector function but also the neighboring WT Treg cells in the Neuritin 1-dependent manner. Although it still requires further validation and investigation of the impact of Neuritin 1 on driving Treg cell expansion and effector function, nevertheless, our findings uncover a previously underappreciated role of a neuronal protein in regulating Treg cell suppressive function and its potential in treating allergies and autoimmune diseases.

In summary, accumulating evidence has established miRNAs as important molecular determinants that regulate many aspects of Treg cell biology (Cho et al., 2021; Lu & Liston, 2009; Lu & Rudensky, 2009). Nevertheless, our understanding of miRNA-dependent control of Treg cell-mediated immune regulation remains limited. Moving forward, besides continuing the current efforts to identify new miRNAs and their targets in Treg cells, additional attention should be paid to understand the molecular mechanisms underlying the regulation and function of miRNAs prevalently expressed in distinct Treg cell subsets and their corresponding interacting partners as well as to further examine how the expression of individual miRNAs within the same the miRNA family can be differently regulated under certain circumstances to optimally control a particular type of Treg cell response.

Chapter 4, in part, includes parts of an adapted version of the material published in Immunological Reviews. Cho, S.*, **Dong, J.***, & Lu, L. (2021). Cell-intrinsic and -extrinsic roles

of miRNAs in regulating T cell immunity. Immunological Reviews, 304(1), 126-140. *denotes co-first authorship. This dissertation author was the co-first author of the article.

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