UNIVERSITY OF CALIFORNIA, SAN DIEGO

Excessive miR-27 Expression Impairs Regulatory T cell-mediated Immunological Tolerance

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by

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The Dissertation of Leilani Ong Cruz is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

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DEDICATION

I dedicate this dissertation to my loving parents, Estrellita and Dionisio, who have sacrificed so much to give me all the opportunities in order for me to succeed. I could have not accomplished all my dreams and goals without your unconditional support and belief in me.

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ABSTRACT OF THE DISSERTATION

Excessive miR-27 Expression Impairs Regulatory T cell-mediated Immunological Tolerance

by

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The immune system has evolved to mount effective responses to numerous and enormously diverse microbial pathogens. Specifically, T cells are able to respond to different environmental cues and differentiate into distinct helper T (Th) subsets through acquiring "master transcription" factors and secreting distinctive cytokines for proper host defense. However, immune activation caused by immune responses against self and foreign antigens need to be tightly controlled to prevent overt inflammation and tissue damage. To this end, a specific subset of T cells, so-called regulatory T (Treg) cells is absolutely necessary for the negative regulation of T cell-mediated immune responses. Several studies show the loss of Treg-mediate regulation can lead to the lost of peripheral tolerance, and the onset of

different autoimmune diseases. Additionally, discovery of microRNAs (miRNAs) have been demonstrated to mediate immune regulation through post-transcriptional gene regulation. Direct binding of miRNA at the 3' untranslated region located on the mRNA targets leads to mRNA degradation.

Of interest, miR-23˜27˜24 cluster contains a unique expression profile with differentially low expression in conventional T (Tconv) cells while maintaining high levels in Treg cells. We hypothesized low expression of this miRNA family is necessary for proper effector T cell function at both physiological and pathological settings. We show that the miR-23˜27˜24 clusters are capable in regulating T cell biology in a collaborative manner, particularly Th2 immunity. Previously, elevated miR-27 expression in T cells isolated from multiple sclerosis patients has been suggested to facilitate disease progression through inhibiting Th2 immunity and promoting Th1 responses. We demonstrate that while mice with T cell-specific overexpression of miR-27 harbor dysregulated Th1 responses and develop autoimmune pathology these disease phenotypes are not driven by miR-27 in effector T cells in a cell-autonomous manner but rather result from a severely compromised Treg cell compartment. Mechanistically, miR-27 represses several known as well as previously uncharacterized targets that play critical roles in controlling multiple aspects of Treg cell biology. Collectively, our findings identify miR-23˜27˜24 clusters to have important immunological roles in effector function and Treg cell-mediated immunological tolerance.

INTRODUCTION

To mount effective immune responses to numerous diverse microbial pathogens, the adaptive immune system generates tremendous diversity in its recognition molecules allowing for the recognition of billions of distinct structures. During infection, naive $CD4^+$ T cells undergo clonal expansion and differentiate into functionally distinct Th subsets upon activation by antigen presenting cells (APCs) for proper host defense against different types of foreign invaders. These Th subsets facilitate different types of immune responses through the production of defined sets of effector cytokines. However, dysregulated Th responses and cytokine production can also lead to the development of Th- associated autoimmune diseases. Therefore, immune activation needs to be tightly controlled to prevent unwanted inflammation and tissue damage. To this end, regulatory T (Treg) cells have emerged as a dedicated immune population crucial for the negative regulation of immune responses. Treg cells that develop in the thymus and those that are induced in the peripheral tissues control effector T cell responses by producing immunosuppressive cytokines and the loss of Treg-mediate regulation can lead to a breakdown in peripheral tolerance. In addition to the aforementioned cellular players in governing the outcome of the immune responses, a class of short regulatory non-coding RNAs termed microRNA (miRNA), have been show to play a role in organ development, cellular differentiation, homeostasis, and function, and have also been shown to be pivotal in immune regulation. Selective expression of a defined subset of miRNA in each T cell subset is suggested to have distinct roles of miRNA in

controlling specific T cell differentiation and function. This dissertation expands upon the current understanding of the emerging role of miR-23˜27˜24 cluster (miR-23 cluster) in T cell immunity and Treg cell-mediated immunological tolerance.

$CD4^+$ T and Treg Cell Biology

In the past two decades, the field of immune regulation has become more expansive and tremendously complex. It has been shown that the immune system has evolved to mount an effective response to protect us from numerous and enormously diverse microbial pathogens. Specifically, T cells are highly specialized to defend against a wide range of bacterial and viral infections, and can also mediate immune surveillance against tumor cells and react to foreign tissues with a unique antigen-binding molecule, the T-cell receptor (TCR) located on the cell surface. The process of T cell development and maturation consists of a series of developmental stages beginning in the bone marrow. T cell progenitors originate in the bone marrow and migrate to the thymus where they continue to complete their maturation process.

At the earliest stage, immature thymocytes express neither CD4 nor CD8 molecules on their surfaces and are classed as double-negative (DN) cells. The next phase developing thymocytes enter is the double-positive (DP) stage where they express both CD4 and CD8 as well as functional TCRs on their cell surface. DP thymocytes that interact with major compatibility complex (MHC) class I or II appropriately will receive the survival signal and be positively selected. The cells that interact with MHC class II molecules will eventually become CD4 single-positive (SP) cells while those that interact with MCH class I molecules will mature into CD8SP cells by down-regulating either CD8 or CD4 expression respectively. In the last step of T cell development, a process of negative selection occurs resulting in the removal of thymocytes expressing TCRs that strongly interact with 'self' peptides presenting

on the MHC molecules. The remaining thymocytes exit the thymus as mature naive T cells and can circulate in the secondary lymphoid organs in anticipation of recognizing foreign antigens.

$CD4^+$ T cells play central roles in the immune system. They help B cells make high affinity antibodies, enhance and maintain the response of $CD8^+$ T cells, regulate macrophage function and orchestrate immune responses against various pathogenic microorganisms. Upon activation through interacting with APCs, naive $CD4^+$ T cells differentiate into functionally distinct Th subsets for proper host defense against different types of foreign invaders. These Th subsets exert their immune function through the production of defined sets of effector cytokines. For example, infection of intracellular microorganisms elicits a type 1 inflammatory response characterized by a marked increased of IFNγ-secreting Th1 cells driven by the transcription factor, T-bet. In the event of parasitic infection, IL-4 drives the induction of Gata3, the master transcriptional regulator of Th2 cells. The differentiated Th2 cells further promote the production of IL-4 as well as IL-5 and IL-13 [100]. Recently a third Th subset, Th17 cells has been identified. The differentiation of Th17 cells is driven by the expression of orphan retinoid receptor, RORγt. These cells secrete IL-17 as their signature cytokine and are required to control extracellular bacterial, fungal and viral infections at mucosal sites.

Although the majority of self-reactive T cells are eliminated during negative selection in the thymus, such mechanisms of tolerance, operating in a cell-intrinsic manner, appear insufficient to counter the threat of immune-mediated pathology. As such, dysregulated Th1 and Th2 responses can lead to the development of autoimmune diseases such as Type 1 diabetes, asthma, and allergic disorders, while uncontrolled Th17 response has been linked to many chronic autoimmune inflammation diseases including multiple sclerosis as well as rheumatoid arthritis. In the past decade, a unique mechanism of negative regulation of immune responses by a dedicated population of so-called Treg cells has become a focus

of intensive investigation [110]. During T cell development, some CD4SP thymocytes can acquire Foxp3 [30, 43, 54], a protein encoded by an X-chromosome-linked protein that serves as a lineage specification factor for Treg cells. Loss-of-function mutations in Foxp3 in humans leads to a severe early fatal multi-organ autoimmune disorder known as IPEX syndrome and a similarly devastating autoimmune disease phenotype can be reproduced in mice with Foxp3 mutations (scrufy) or deletion.

While the majority of Foxp3$^+$ Treg cells are generated in the thymus, termed 'natural' Treg (nTreg) or thymic-derived Treg (tTreg) cells, mounting evidence show CD4$^+$ CD25$^-$ conventional T (Tconv) cells can be converted into mature and functional Treg (iTreg) cells by acquiring Foxp3 expression and immunosuppressive properties upon TGFβ stimulation in the periphery [14]. In addition to Foxp3, many other molecular players have been demonstrated to be crucial in controlling Treg cell development and maintaining Treg cell lineage stability. For example, loss of Foxo1 in T cells resulted in reduced Treg cell numbers and mice with T cell-specific Foxo1 ablation developed multiorgan autoimmunity including exocrine pancreatitis [52]. Moreover, several studies have demonstrated the expression of Runx1 (Runt-related transcription factor 1) is crucial for maintaining Foxp3 expression in Treg cells through forming a complex with CBFβ [96]. Mice lacking CBFβ exclusively in Treg cell lineage harbored Treg cells with reduced amounts of Foxp3. As a consequence, these Treg cells could not properly function and mice developed lymphoproliferative syndrome over time [109]. Finally, c-Rel, a member of NFκB family was shown to drive thymic Treg cell development by promoting the formation of a Foxp3$^-$ specific enhanceosome [106]. In addition, c-Rel is also required for optimal homeostatic expansion of peripheral Treg cells [45] as well as iTreg induction in the periphery [126]. Together, many key Treg cell-associated molecules cooperate with Foxp3 to ensure proper Treg cell development in order to maintain immunological tolerance [97].

Despite rapidly accumulating knowledge of Treg cell biology, our understanding

of the precise molecular mechanisms underlying Treg cell-mediated suppression remains incomplete. Initially, it was suggested that Treg cells inhibit TCR-induced proliferation and IL-2 transcription of Tconv cells in a cell-to-cell contact dependent manner. Later, Treg cells were shown to be capable of suppressing Tconv cell expansion through the secretion of immunosuppressive cytokines such as TGFβ, IL10, and IL-35. TGFβ production by Treg cells was shown to be necessary to prevent colitis *in vivo* [46,103] even though TGFβ-deficient Tregs could suppress Tconv proliferation *in vitro*. Similarly, IL-10 also plays an important role in Treg cell-mediated suppression of intestinal inflammation. In a T cell transfer colitis model, co-transfer IL-10-deficient Treg cells or administration of IL-10 neutralizing abs in mice transferred with WT Treg cells both led to uncontrolled effector T (Teff) cell activation and resultant tissue damage [1]. In addition to its role in controlling intestinal inflammation, IL-10 has also been implicated in Treg cell-mediated immune regulation in other disease settings such as infections and EAE [4, 84]. More recently, IL-35, a novel immunosuppressive cytokine was shown to play a pivotal role in conferring suppressor function to Treg cells [23]. Treg cells devoid of IL-35 exhibited reduced suppressive ability in maintaining gut homeostasis. Lastly, while GZMB is more prevalent in the context of natural killer (NK) and $CD8^+$ T cell during an immune response, mounting evidence has shown in both mice and humans, that GZMB plays a key role in Treg cell-mediated immune regulation under different physiological and pathological settings [12].

In the last decade, considerable effort has been put into identifying novel molecular processes necessary for proper Tconv and Treg cell differentiation and function. Discovery of miRNAs is one of the recent major scientific breakthroughs that has impacted the immunology field. miRNAs have been implicated in the regulation of maturation, proliferation, differentiation and activation of immune cells and their impact on T cell immunity will be discussed below.

MicroRNA

Recent studies of miRNAs, a subset of short non-coding RNA transcripts (˜22 nts) that could repress gene expression post-transcriptionally, have uncovered a new level of complexity in the regulation of development and function of the biological system [93]. Following the expression of the primary transcript in the nucleus, nuclear processing by an RNase III molecule, Drosha produces a pre-miRNA transcript [8], which can be shuttled into the cytoplasm by exportin 5 [40, 65, 140]. In the cytoplasm, another RNase III molecule, Dicer, further processes the pre-miRNA hairpin structure into a 19- to 24-base-pair duplex product, which contains both the mature miRNA and its antisense strand. The star (or antisense) strand is degraded while the guide strand containing the mature miRNA sequence is capable of being incorporated into the RNA-induced silencing complex (RISC), which contains another core component, Argonaute (Ago) protein [53, 71]. The miRNA can guide the RISC to the 3'-untranslated site of its target mRNA through the 'seed sequence' located at positions 2 to 7 from the 5'end to match the complementary base sequence found on the mRNA targets. Through direct degradation or translational inhibition of their mRNA targets, these small regulatory RNA molecules can alter the basal state of immune cells as well as the outcome of stimulatory events.

To date, many groups have demonstrated that miRNAs are pivotal in regulating both adaptive and innate immunity, including controlling the differentiation of various immune cell subsets as well as their immunological functions [79]. Aberrant miRNA expression patterns have been documented in a broad range of human diseases [16]. The role of miRNA in T cell immunity was first demonstrated by a study where the whole miRNA network was deleted in T cells. To this end, T cells lacking Dicer exhibited preferential Th1 induction under non-polarizing conditions, and displayed significant impairments in Th17 induction and TGFβ-mediated Treg cell induction [90]. Further studies revealed distinct sets of miRNAs

that regulate the differentiation and function of different Th lineages further suggesting a pivotal role of miRNA in T cell immunity. To this end, deficiency in miR-155 promoted Th2 differentiation while Th1 and Th17 responses were attenuated. In contrast, both miR146a and miR-29 have been shown to function as negative regulators of Th1 responses. While miR-146a controls IFNγ signaling through targeting Stat1, miR-29 can inhibit Th1 differentiation by targeting T-bet [117], Eomes, and IFNγ directly. Moreover, miR-326 was shown to enhance Th17 differentiation by targeting Ets-1, and its overexpression led to an elevated number of Th17 cells in mice along with a severe EAE disease phenotype [26]. As for T follicular helper (Tfh) cells, 2 members, miR-17 and miR-92 of the miR-17˜92 family were shown to both target the Phlpp2 gene. This study also illustrated the ability of different members of a given miRNA family in cooperatively regulating a defined T cell response [51]. Finally, previous studies have demonstrated two key miRNAs, miR-155 [80] and miR-146a [77] that are critical for maintaining normal Treg cell homeostasis, and their suppressor function to control Th1 inflammation, respectively.

The miR-23˜27˜24 Clusters

While the aforementioned studies have demonstrated crucial roles of miRNA in T cell differentiation, the highly diverse and distinct patterns of miRNA expression in each T cell subset imply that further studies are needed to comprehensively define the role of individual miRNAs in controlling the development and function of different T cell subsets at steady state but also in clinically relevant disease settings [77, 105]. Among those miRNAs that are differentially expressed in Treg cells vs. Tconv cells, miR-23a and miR-23b clusters are of particular interest. Both miR-23a and b clusters (miR-23a/b) were highly up-regulated in Treg cells [21, 48]. Moreover, upon activation the differences in miR-23a/b clusters expression between Treg cells and Tconv cells further increased as they were further up-regulated in

Treg cells while down-regulated in Tconv cells, suggesting this miRNA family might play distinct roles in controlling Treg cells and Tconv cells, respectively.

miR-23a cluster is comprised of three individual miRNAs; miR-23a, miR-27a, and miR24-2, localized on chromosome 8 while its paralog miR-23b (miR-23b˜27b˜24-1) cluster is localized on chromosome 13. The members of each cluster are transcribed on the primary transcript before being processed into mature miRNAs. Mature sequences of miR-23a and miR-27a differ by just one nucleotide in comparison to their paralogs miR-23b and miR-27b respectively, while miR-24-1 and miR-24-2 share the same mature sequence suggesting overlapped gene targeting in the same cell-types. On the other hand, even though different paralogical miRNAs might share common targets, the regulation of the expression of these two clusters is likely independent from each other as they reside in two different chromosomes, moreover, even within the same miRNA cluster, depending on different biological conditions, the expression pattern of different members can also vary. To this end, it is well documented that in some conditions, all three members of the miR-23a cluster have similar expression pattern while in other conditions only one or two miRNAs are specifically expressed [16].

Elevated expression of both miR-23a/b clusters has been reported to be associated with many human solid tumors as well as hematopoietic malignancies [20,124,127,135,139]. It was shown that they are able to contribute to oncogenesis by promoting angiogenesis, cell cycle progression or negatively regulating apoptosis through the repression of many targets that are essential for controlling aforementioned biological processes [15, 17, 39, 62, 85]. In addition to cancer-related studies, miR-23 has been implicated in cardiac hypertrophy and muscular atrophy. A cardiac hypertrophy study reported miR-23 as a pro-hypertrophic miRNA that can be regulated by the nuclear factor of activated T cells (NFATc3) transcription factor [70]. Moreover, miR-23 plays a substantial role in protecting against skeletal muscle atrophy as it can repress the expression of MAR/bx/atrogin-1, a muscle-specific E3 ubiquitin ligase [128]. While miR-24 has been extensively studied in tumorigenesis, miR-24 has also been studied

in hematopoietic differentiation through targeting human activin type I receptor ALK4 (hALK4), a molecule crucial for erythropoiesis [132]. In addition, miR-24 also appeared to play a central role in modulating vascular smoother muscle-cell (vSMC) during vascular development and vascular remodeling through targeting the platelet-derived growth factor BB (PDGF-BB) and TGFβ signaling pathways. Finally, in addition to its role in tumor biology [35, 39, 85], miR-27 was reported to be involved in in regulating fat metabolism [49]. miR-27 could serve as a negative regulator of adipocyte differentiation through blocking the transcriptional induction of proliferators-activated receptor-gamma (PPARγ) [55, 68].

Despite the fact that the miR-23a/b families have primarily been studied for their impact on various cancer models and other human diseases, current understanding of how this miRNA family affects T cell biology has yet to be fully explored. *In silico* target analysis of the individual miRNAs within miR-23 clusters has suggested a potential important role of miR-23 clusters in controlling T cell responses. To this end, miR-27 is of particular interest as many previously characterized miR-27 targets such as Foxo1 and Runx1 are in fact also well recognized for their roles in controlling T cell development and function [5, 91]. Additionally, several Smad proteins, key molecules of TGFβ signaling, have been shown to be targeted by miR-27 [104]. As TGFβ signaling is crucial for both Th17 and iTreg differentiation, it is possible that miR-27-mediated Smad repression could impact Th17 and iTreg cell responses. Finally, a previous study has reported that miR-27 is highly expressed in $CD4^+$ T cells isolated from multiple sclerosis patients. It was suggested that miR-27 promoted proinflammatory IFNγ responses through targeting BMI1, a molecule that stabilizes Gata3, illustrating the biological significance and possible therapeutic potential of targeting miR-27 in human autoimmune diseases [36].

In this dissertation, by employing both gain- and lost-of-function approaches as well as performing different biochemical and immunological studies, we demonstrate that miR-23a/b clusters regulate multiple aspects of T cell biology. In particular, miR-24 and

miR-27 cooperatively restrain type II immunity by targeting several Th2-associated genes. Further, we show miR-27 acts as a central regulator in Treg cell development and function. The autoimmune pathology observed in mice with excessive expression of miR-27 in T cells is not driven by miR-27 in Teff cells in a cell-autonomous manner, rather it was the result of a perturbed Treg cell compartment. Finally, we discuss the importance of the miR-23a/b cluster as an immune regulatory miRNA family that plays a diverse role in controlling T cell immunity and Treg cell-mediated immunological tolerance. Our findings not only establish the miR-23a/b clusters as key players in regulating T cell biology but also provides a solid foundation for future studies to explore this miRNA family in other aspects of immune regulation.

Chapter 1

MiR-23˜27˜24 clusters control effector T cell differentiation and function

1.1 Abstract

Coordinated repression of gene expression by evolutionarily conserved microRNA (miRNA) clusters and paralogs ensures that miRNAs efficiently exert their biological impact. Combining both loss- and gain-of-function genetic approaches, here we show that the miR-23˜27˜24 clusters regulate multiple aspects of T cell biology, particularly Th2 immunity. Low expression of this miRNA family confers proper effector T cell function at both physiological and pathological settings. Further studies in T cells with exaggerated regulation by individual members of the miR-23˜27˜24 clusters revealed miR-24 and miR-27 collaboratively limit Th2 responses through targeting IL-4 and GATA3 in both direct and indirect manners. Intriguingly, while overexpression of the entire miR-23 cluster also negatively impacts other Th lineages, enforced expression of miR-24, in contrast to miR-23 and miR-27, actually

promotes the differentiation of Th1, Th17 and iTreg cells, implying that under certain conditions, miRNA families can fine tune the biological effects of their regulation by having individual members antagonize rather than cooperate with each other. Together, our results identify a miRNA family with important immunological roles and suggest tight regulation of miR-23˜27˜24 clusters in T cells is required to maintain optimal effector function and to prevent aberrant immune responses.

1.2 Introduction

Upon microbial insult, T cells differentiate into effector helper T (Th) cells to generate protective immune responses. Recently, a class of short regulatory non-coding RNAs, the so-called microRNAs (miRNAs), known for their role in tissue development, cellular differentiation and function, have been demonstrated to be pivotal in regulating immune responses [64, 94]. Selective expression of a defined set of miRNAs in each T cell lineage suggested miRNAs play distinct roles in controlling different aspects of T cell immunity [61]. However, an emerging view purports that miRNAs, rather than enacting drastic gene changes, primarily reinforce pre-existing transcriptional programs or buffer against stochastic fluctuations in gene expression [27]. Indeed, despite complex biological phenotypes observed in mice with total T cell- or regulatory T (Treg) cell-specific inactivation of the entire miRNA pathway [19, 21, 73, 145], the analysis of individual miRNA contribution to specific T cell responses has been largely restricted to a select few whose deficiency resulted in pronounced perturbation of immune cell function [60].

Many known miRNAs exist in clusters and paralogs with high degrees of evolutionary conservation, suggesting a means for increasing miRNA's impact on gene regulation and resultant biology. The miR-17˜92 cluster, for example, controls immune responses through several cluster members that either target the same gene or different components of common

biological pathways [3, 51, 116, 125]. Like the miR-17˜92 family, the miR-23˜27˜24 family contains multiple members and two paralogs: miR-23a˜27a˜24-2 (miR-23a cluster) on chromosome (chr) 8 (chr 19 in human) and miR-23b˜27b˜24-1 (miR-23b cluster) on chr 13 (chr 9 in human). Mature sequences of miR-23a and miR-27a differ by just one nucleotide in comparison to their correseponding paralogs miR-23b and miR-27b while miR-24-1 and miR-24-2 share the same mature sequences. However, despite their distinct expression patterns in T cells, to date studies of the miR-23 clusters have primarily focused on their role in tumorigenesis [17, 39, 82, 85, 131, 141]. Even when *in silico* target analysis of the individual miRNAs within the miR-23 clusters has suggested an important role for this miRNA family in controlling T cell responses [16], direct experimental evidence in this direction remains limited [13, 36, 69].

In this study, by employing both gain- and loss-of-function genetic approaches, we investigated the roles of the miR-23 clusters as well as each miRNA member within this miRNA family in T cell biology. Enforced expression of this miRNA family in T cells resulted in dysregulated T cell activation and autoimmune inflammation whereas its ablation in T cells led to reduced proliferation and activation even in response to immune challenges. Moreover, in addition to having a general impact on T cell activation, the miR-23 clusters play a central role in T cell differentiation. In particular, different members of the miR-23 family cooperate to potently control Th2 immunity by targeting IL-4 and GATA3 in both direct and indirect manners. Mice harboring T cells devoid of miR-23 clusters developed exacerbated airway eosinophilic inflammation and mucus hypersecretion upon allergen sensitization and challenge. Interestingly, in modulating the differentiation of other Th lineages, we also found that different members of this miRNA family could antagonize rather than cooperate with each other. Together, our data identify a new "immune regulatory" miRNA family that plays diverse roles in controlling T cell immunity.

1.3 Results

Enforced expression of the miR-23 cluster in T cells leads to spontaneous lympho-hyperactivation phenotypes

To determine the role of the miR-23 clusters in T cell immunity, we first confirmed the expression levels of both miR-23 clusters in T cells by comparative miRNA expression profiling. Consistent with previous reports, [21, 48] higher levels of miR-23 family members were detected in Treg cells compared to conventional T (Tconv) cells regardless of activation status (Figure 1.1). A recent work has shown that miR-23a downregulation promotes effector $CD8^+$ T cell differentiation and cytotoxicity [69]. Moreover, it was previously demonstrated that global miRNA downregulation in T cells resulted in the de-repression of genes critical for T cell differentiation and effector functions [8]. Therefore, it is possible that the miR-23 clusters are expressed at necessarily diminished levels to confer normal effector function to Tconv cells. To determine the impact of exaggerated miR-23 cluster regulation on T cell immunity, we generated mice that selectively overexpress the whole miR-23a cluster (R23CTg) or individual members (R23Tg, R24Tg or R27Tg) in T cells (Figure 1.2). As shown in Figure 1.3A-E, significantly reduced thymic Treg cell numbers were detected in R23CTg mice at 6-8 weeks of age while no significant alteration in peripheral Treg cell frequencies nor in total thymic or splenic cellularities could be observed. These results suggested that although the miR-23 clusters are highly expressed in Treg cells, enforced expression of this miRNA family prior to Treg cell differentiation might impair their development in the thymus.

Interestingly, R23CTg mice exhibited unrestrained activation of effector T cells with greater Ki67 staining, increased $CD44^{hi}CD62L^{lo}$ cell subset, and elevated IFNγ production (Figure 1.3F-H), despite harboring normal numbers of peripheral Treg cells with comparable suppressor function (Figure 1.3I). These results support our initial hypothesis that expression

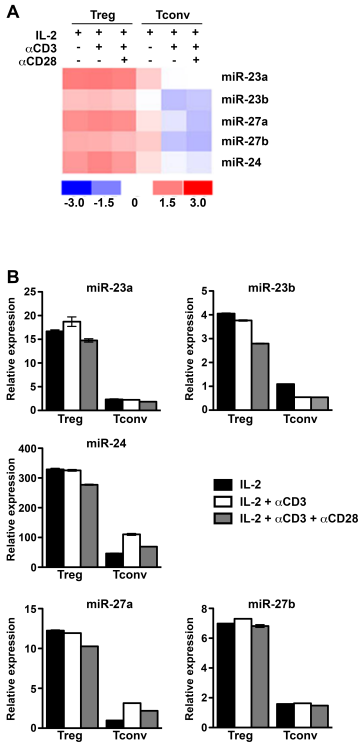

Figure 1.1: **Distinct expression patterns of miR-23 clusters in Treg verus T conv cells** miRNA array (A) and qPCR analysis (B) of the expression of individual miRNA members in miR-23 clusters in T reg cells versus T conv cells under different culture conditions. Data are representative of two independent experiments. n = 6-15.

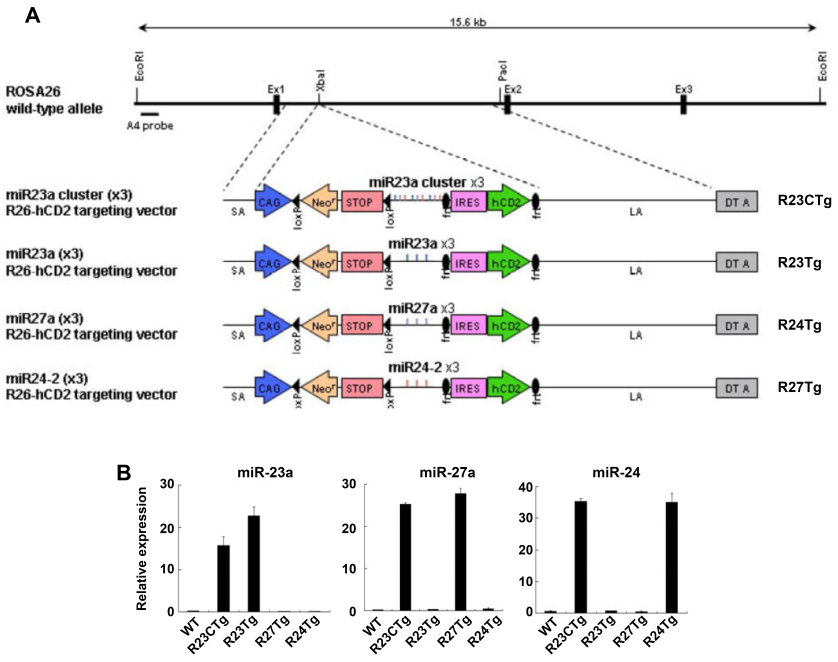

Figure 1.2: **Conditional overexpression of miR-23a cluster in T cells** (A) Schematic representation of the targeting strategy for ROSA Tg. The resultant mice were bred to $CD4^{cre}$ mice to overexpress total or individual members of miR-23a cluster in T cells, respectively. (B) qPCR analysis of relative expressions of miR-23a, miR-27a and miR-24 in T cells isolated from different ROSA Tg mice. Data are representative of four independent experiments. (n=4-8).

of miR-23 clusters at a particular reduced level is required to maintain proper effector T cell activation and function. Finally, as mice aged, the T cell activation phenotypes in R23CTg mice could be mostly recapitulated when miR-27 alone and to a lesser degree when miR-24 alone were overexpressed, while effector T cell responses in R23Tg mice were largely unaltered (Figure 1.3J).

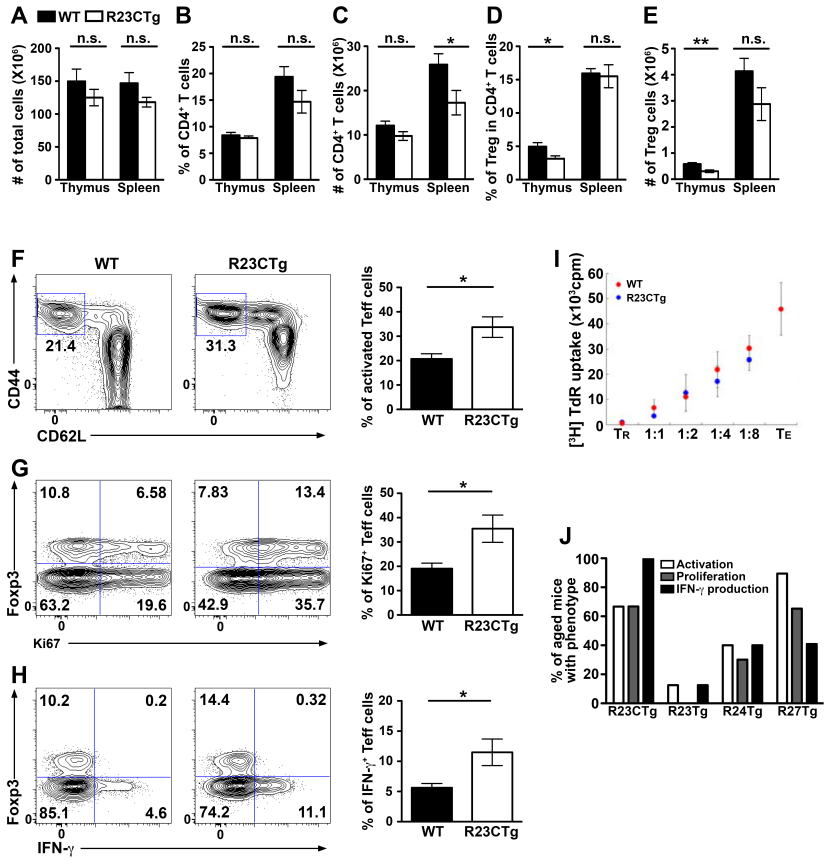

Figure 1.3: **Enforced expression of the miR-23 family in T cells resulted in dysregulated T cell activation and cytokine production in mice.** (A-E) Cellularity of the thymus and spleen and the proportions and absolute numbers of thymic and splenic total $CD4^+$ T cells and $Foxp3^+CD4^+$ T reg cells in R23CTg mice and control littermates are shown. FACS analysis of $CD44^{hi}$ $CD62L^{lo}$ subset (F), $Ki67^+$ (G), and IFNγ -secreting cells (H) in $Foxp3^-$ $CD4^+$ effector T cells in spleen from 6-8-wk-old R23CTg mice and control littermates were shown. Data are representative of four independent experiments. n = 6-10. *, P <0.05; **, P <0.01. (I) Treg cells (TR) isolated from R23CTg or WT control littermates were subjected to *in vitro* suppression analysis at indicated ratios of responder T cells (TE). Data are representative of three independent experiments. n = 6. (J) Percentages of mice (˜5 mo of age) with overexpression of the whole cluster or individual members harboring effector T cells displaying increased activation, proliferative activity, and production of IFNγ. n = 4-17.

miR-23 family controls the differentiation of multiple Th cell lineages

Having observed miR-23 cluster-dependent impairment in T cell immunity under steady state, we examined the effects of overexpression of the miR-23 clusters on effector T cell differentiation. Interestingly, despite T cell hyperactivation in R23CTg mice, overexpression of the miR-23 cluster negatively impacted the differentiation of every helper T (Th) lineage tested (Figure 1.4A, B). Cluster overexpression also compromised the generation of TGFβ-induced Treg (iTreg) cells, just as it did thymic Treg development (Figure 1.3A). Further studies demonstrated that miR-27 controls the generation of all Th lineages in the same manner as the whole miR-23 cluster (Figure 1.4C-F). On the other hand, overexpression of miR-23 only led to impaired iTreg and Th17 cell induction without any significant effect on Th1/Th2 polarization. Finally, overexpression of miR-24 reduced Th2 but promoted Th1, Th17 and iTreg differentiation (Figure 1.4C-F). Collectively, our studies suggested that while individual miR-23 family members can cooperatively regulate the same biological processes, they can also antagonize each other under certain conditions to fine tune the biological effects resulting from the regulation of the entire miRNA family.

miR-24 and miR-27 regulate Th2 differentiation and function through targeting IL-4 and GATA3

Given that only Th2 cells were not controlled contrastingly by individual members of the miR-23 family, we decided to further investigate the molecular mechanisms underlying miR-23 cluster-dependent regulation of Th2 immunity. The fact that overexpression of the miR-23 cluster in T cells reduced not only the frequency of IL-4 secreting cells but also the amount of IL-4 being produced on a per cell basis suggested that IL-4 itself might be a direct target of this miRNA family (Figure 1.5A, B). Analysis of previous results from high-throughput sequencing of RNAs isolated by cross-linking immunoprecipitation (HITS-CLIP) showed a miR-24 binding site on the relatively short 3'UTR of the IL-4 transcript [75] (Figure

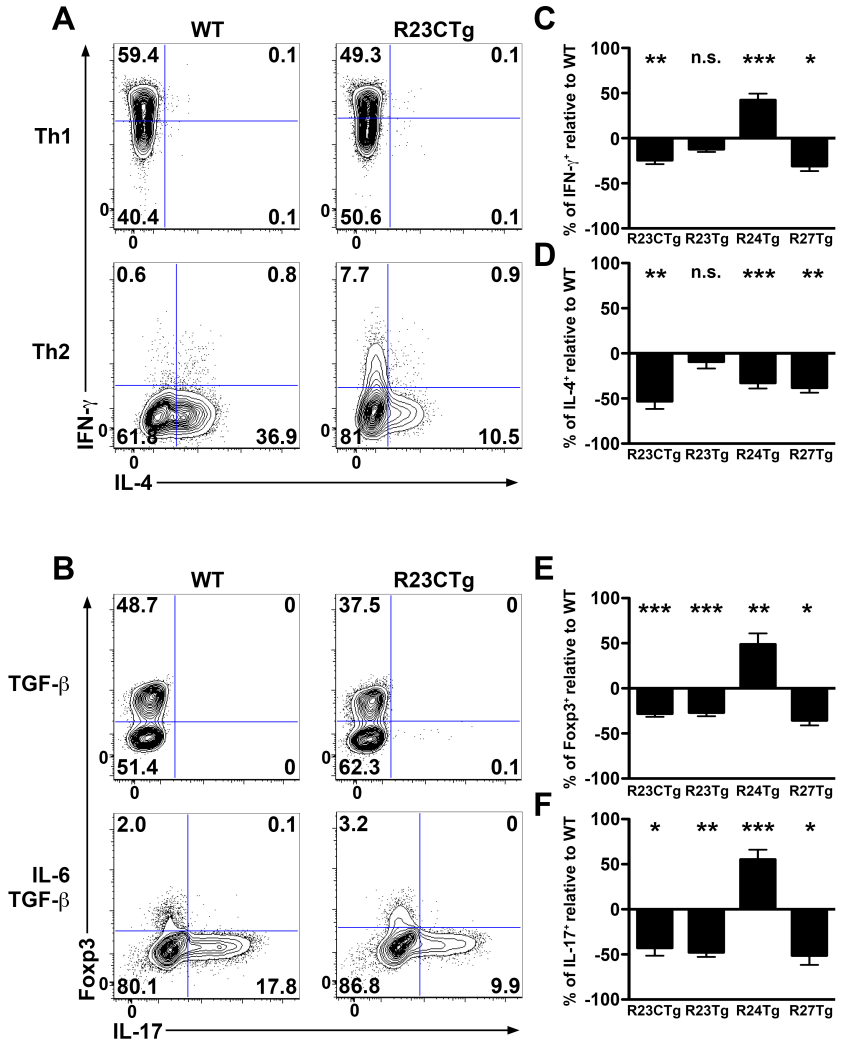

Figure 1.4: **miR-23 family controls the differentiation of multiple T cell lineages.** Naive T cells isolated from R23CTg mice and WT littermates were cultured *in vitro* under Th1/Th2-polarizing (A) and Th17/iTreg differentiation (B) conditions, respectively. IFNγ, IL-4, IL-17, and Foxp3 staining were assessed by FACS analysis. Percent- ages of cytokine-specific intracellular staining of the T cells isolated from mice with overexpression of the whole miR-23 cluster, as well as individual members under (C) Th1-, (D) Th2-, (E) iTreg-, and (F) Th17-polarizing conditions. Data are representative of three independent experiments. n = 3-6. *, P <0.05; **, P <0.01; ***, P <0.001.

1.5C). More interestingly, this putative binding site is located just one nucleotide after the stop codon, strongly suggesting the 3' end of miR-24 would interact with sequences in the coding region of IL-4 mRNA (Figure 1.5D). While most of the miRNA studies to date have been focused on the regulatory effect of miRNAs in the 3' UTR of the target genes, it was well documented that miRNAs could also inhibit their target protein translation potentially through interfering with ribosome movement [9,41]. Our luciferase reporter results confirmed IL-4 is indeed a direct target of miR-24. Cotransfection of miR-24 with the IL-4 3'UTR alone or extending partly into the IL-4 coding region resulted in appreciable repression of reporter activity, whereas mutation of the miR-24 binding site abolished this repression (Figure 1.5E). Moreover, the consistent increase in repression by miR-24 detected when the construct included part of the IL-4 coding region (C2) compared to the one without (C1) further suggested that interaction between miR-24 and the IL-4 coding region positively contributed to miR-24-mediated gene regulation (Figure 1.5E). Consistent with these findings, R24Tg T cells produced diminished amounts of IL-4 similar to what was observed in R23CTg T cells (Figure 1.5F, G). Yet negative regulation of IL-4 by miR-24 could not explain the reduced IL-4 production and impaired Th2 responses observed in T cells that overexpressed miR-27 alone. Further analysis of HITS-CLIP results revealed that GATA3, a central transcription factor required for Th2 differentiation and cytokine production, is a potential target of miR-27. The 3'UTR of GATA3 contains three putative binding sites for miR-27, as well as one for miR-24 (Figure 1.5H). Consistent with this finding, compared to WT, GATA3 protein levels were significantly reduced in $CD4^+$ T cells from R23CTg and R27Tg mice, and to a lesser extent from R24Tg mice, but not from R23Tg mice (Figure 1.5I). Interestingly, luciferase reporter assay demonstrated that GATA3 is directly repressed by miR-27 but not miR-24, and that only the first out of three putative miR-27 binding sites in the GATA3 3'UTR is functional (Figure 1.5J, K). It is likely the reduced GATA3 expression detected in R24Tg T cells is the consequence of reduced autocrine IL-4 signaling [111]. These findings

also evidence that not all binding sites revealed by HITS-CLIP are biologically relevant. Finally, we were able to significantly restore the ability of miR-27-overexpressing T cells to produce IL-4 under Th2 polarizing condition by overexpressing GATA3 (Figure 1.5L-N). Together, these results demonstrated that the miR-23 clusters could suppress Th2 responses through directly repressing IL-4 production as well as inhibiting GATA3-dependent Th2 differentiation program in miR-24- and miR-27-dependent manners.

miR-24 and miR-27 collaboratively modulate Th2 gene network but predominantly repress their targets at the protein level To gain further molecular insights into miR-23 clusters-dependent regulation of Th2 immunity, we performed transcriptome analysis of T cells with overexpression of individual miR-23 family members. $CD25^{-}CD44^{lo}CD62L^{+hi}$naive $CD4^{+}$ T cells were FACS sorted for RNA sequencing to minimize the secondary effects from miR-24- and miR-27-dependent IL-4 and GATA3 repression in differentiated Th2 cells. Consistent with our *ex vivo* observations in the aforementioned different miR-23 family Tg mouse lines (Figure 1.3J), while the global gene expression profile of R23Tg T cells was largely comparable to that of WT, it was most distinct in R27Tg T cells, with R24Tg T cells exhibiting an intermediate pattern (Figure 1.6A). Additional screening of genes associated with Th2 immune responses [119] and GO annotation: http://geneontology.org/) further revealed that a substantial proportion (˜30%; 39/132) of Th2-related genes was already downregulated in non-polarized T cells overexpressing miR-24 or miR-27 (Figure 1.6B). Next, we performed String analysis (http://string-db.org/) to identify potential physical and/or functional interactions between genes that were modulated by miR-24 and/or miR-27 (Figure 1.6C). To this end, many Th2 genes that were downregulated in R24Tg T cells and/or R27Tg T cells were shown to be highly connected to IL-4 and GATA3. Interestingly, both GATA3 and IL-4, as well as Bmi1, a known miR-27 target that could stabilize GATA3 [36], were in fact not downregulated in the corresponding Tg T cells. Among genes that were downregulated, only two, Rap1b and Nfatc2, were predicted to be miR-24 and miR-27

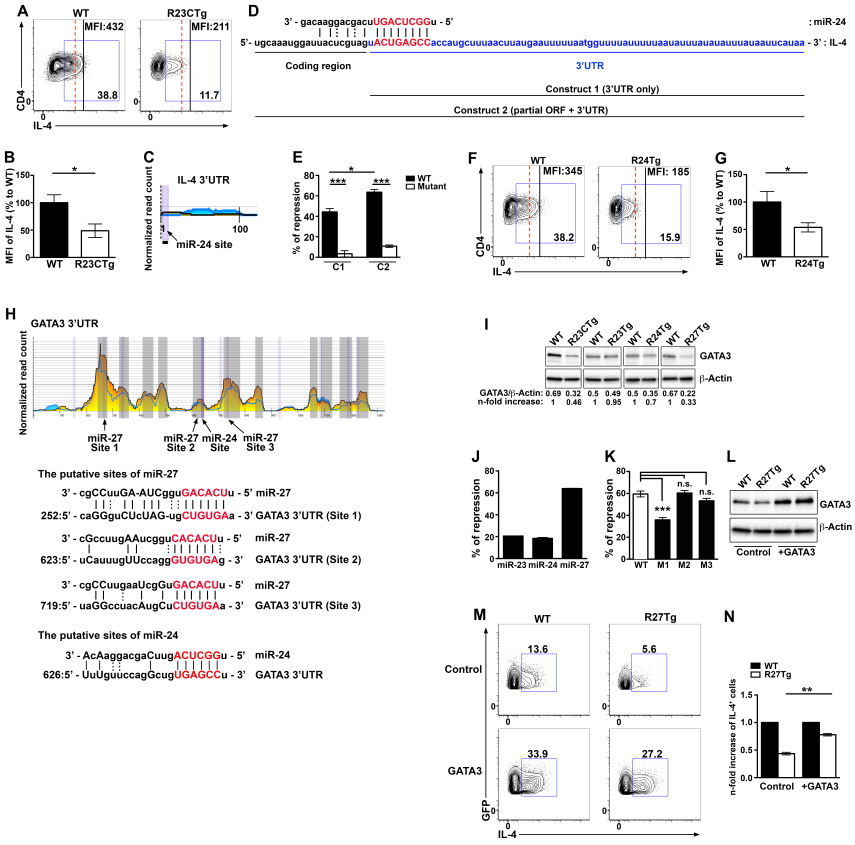

Figure 1.5: **miR-24 and miR-27 target IL-4 and GATA3, respectively.** FACS analysis (A) and ratios of mean fluorescence intensity (MFI; B) of IL-4 from R23CTg $CD4^+$ T cells compared with WT $CD4^+$ T cells under Th2-polarizing condition. HITS-CLIP analysis (C) and sequence alignment (D) of the putative site of miR-24 in IL4. (E) Ratios of repressed luciferase activity of cells with IL-4 3' UTR only (C1) or 3' UTR plus partial coding region containing miR-24-interacting sequences (C2) with or without mutations in the seed sequences in the presence of miR-24 compared with cells transfected with empty vector were shown. FACS analysis (F) and ratios (G) of MFI of IL-4 from R24Tg $CD4^+$ T cells compared with WT $CD4^+$ T cells under Th2-polarizing condition. (H) HITS-CLIP analysis and sequence alignment of putative sites of miR-27 and miR-24 in the 3' UTR of GATA3. (I) Immunoblot analysis of GATA3 expression in T cells cultured for 4 days under Th2-polarizing condition. Densitometric GATA3 expression values were normalized to β-actin expression values and n-fold increase on the basis of each corresponding WT. Ratios of repressed luciferase activity of cells in the presence of WT GATA3 3' UTR transfected with indicated miRNA (J) or WT or mutated GATA3 3' UTR with miR-27 compared with cells transfected with empty vector (K). Immunoblot analysis of GATA3 expression (L) and FACS analysis of IL-4 production (M) in GFP^+ R27Tg $CD4^+$ T cells transduced with GATA3-expressing or control vector with a GFP reporter under Th2-polarizing condition. (N) n-fold increase (on the basis of corresponding WT controls) of $IL\text{-}4^+$ cells in GFP^+ R27Tg $CD4^+$ T cells. All data are representative of three independent experiments. n=3-6.*,$P<0.05$;**,$P<0.01$;***,$P<0.001$.

targets, respectively, by HITS-CLIP analysis (Figure 1.6C). While the functional connection between Rap1b and Th2 cells has not been established except its elevated expression in Th2 cells [119], the role of Nfatc2 in promoting IL-4 production and Th2 differentiation has long been recognized [24, 47]. Therefore, it is plausible that miR-27-mediated Nfatc2 repression could also play an important role in restricting Th2 differentiation and effector function. Nevertheless, the fact that we could only identify one additional miR-23 cluster target that could contribute to the observed Th2 biology by our RNA-seq study and the HITS-CLIP analysis suggested that genes associated with Th2 immune responses are typically not regulated by miR-24 or miR-27 at the mRNA level. In agreement with this hypothesis, cumulative distribution frequency (CDF) plot analysis revealed that there was no detectable difference in the expression of Th2-related genes regardless the presence or the absence of HITS-CLIP-identified miR-24 or miR-27 binding sites in the respective Tg T cell populations compared to control WT T cells (Figure 1.6D). Surprisingly, when all genes were subjected to CDF plot analysis, the ones with HITS-CLIP-identified sites were evidently downregulated when the corresponding miRNA was overexpressed (Figure 1.6E). These results demonstrated that whereas miR-23 family might in general control their targets at the mRNA level, they predominantly repress genes associated with Th2 responses at the protein level.

Enhanced Th2 differentiation albeit reduced activation in T cells devoid of miR-23 clusters led to Th2 associated immunopathology during airway allergic reaction

Thus far, we have shown that exaggerated regulation by the miR-23 clusters in T cells led to impaired Th2 responses. However, it is unclear as to whether elimination of miR-23 cluster-mediated gene regulation would result in enhanced Th2 immunity. To directly address this question and to further study the biological role of the miR-23 clusters, we generated mice harboring either a conditional allele of the miR-23a (miR-23aC^{fl}) or the

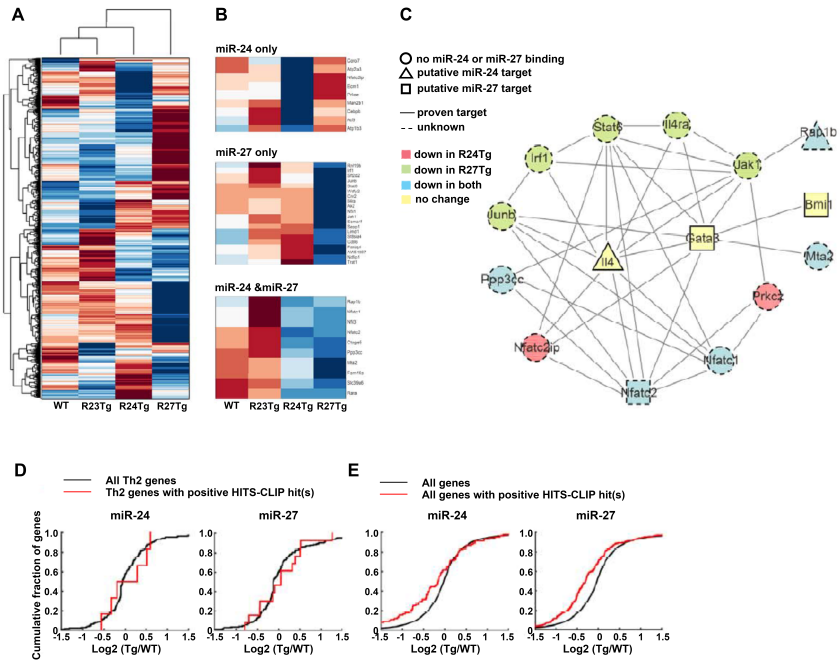

Figure 1.6: **Overexpression of miR-24 or miR-27 negatively impacts Th2 gene network.** (A) Clustering of RNA-seq results from R23Tg, R24Tg, R27Tg, and WT naive CD4$^+$ T cells based on total gene expression. (B) Th2-associated genes that were down-regulated in R24Tg T cells only, R27Tg T cells only, or both were shown. (C) String analysis of potential physical and/or functional interactions between Th2 genes that were proven miR-24 or miR-27 targets or down-regulated by miR-24 and/or miR-27 as shown from the RNA-seq results. Putative targets were defined by having perfect seed complementarity between positions 2 and 7 of the corresponding miRNA with positive Argonaute-binding peaks in the HITS-CLIP dataset. Cumulative distribution frequency plots depicting the effect of overexpression of miR-24 or miR-27 on mRNA expression of Th2-associated (D) or all genes (E). Levels of mRNAs of Th2-associated or all genes bearing predicted binding sites of miR-24 or miR-27 (red line) as defined above were compared with mRNAs of total Th2-associated or all genes (black line).

miR-23b cluster (miR-23bC^{fl}) (Figure 1.7). Despite a lack of aberrant phenotype in mice with T cell-specific ablation of both miR-23 clusters (T-DKO) at steady state (Figure 1.8), unbiased transcriptome analysis revealed ˜94% of genes displaying contrasting expression patterns between T cells isolated from T-DKO vs R23CTg mice (Figure 1.9A). Moreover, when T-DKO T cells were subject to Th2 polarization, significantly more IL-4 producing cells, with increased expression of IL-4 and GATA3 on a per cell basis were detected compared to WT (Figure 1.9B-D), in agreement with our gain of function studies. Finally, while IL-4 and GATA3 were not downregulated in naive T cells by our RNA-seq analysis, we could clearly detect increased mRNA levels of IL-4 and GATA3 in T-DKO T cells and decreased mRNA levels of IL-4 and GATA3 in both R24Tg and R27Tg differentiated T cells upon Th2 polarization (Figure 1.9E, F). Nevertheless, these observed changes in the mRNA levels of IL-4 and GATA3 might be influenced by not only themselves and each other during Th2 differentiation, but also potentially many other genes targeted by miR-24 or miR-27 in T cells.

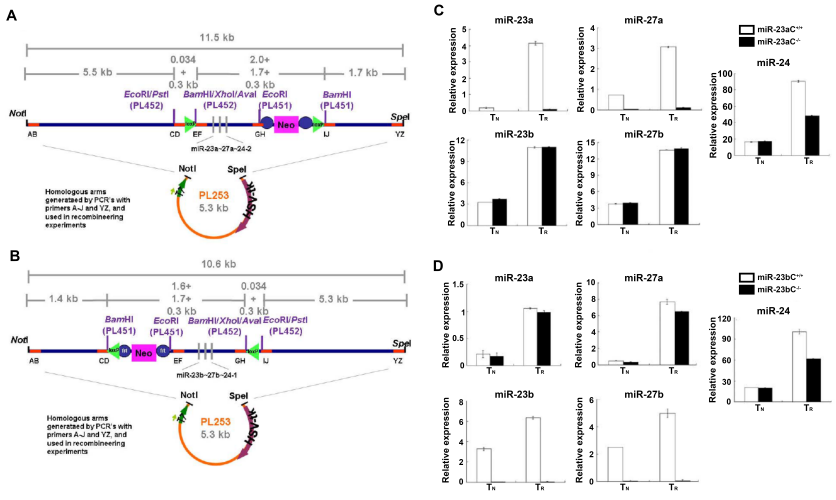

Figure 1.7: . Generation of mice with conditional alleles of miR-23a cluster or miR-23b cluster. Schematic representation of the targeting strategy for (A) miR-23aC^{fl} and (B) miR-23bC^{fl} mice, respectively. Expressions of different miRNA members in miR-23a and miR-23b cluster in Treg cells (TR) and naive T cells (TN) isolated from (C) miR-23aC$^{-/-}$ and (D) miR-23bC$^{-/-}$ mice. Data are representative of two independent experiments. (n=4-6).

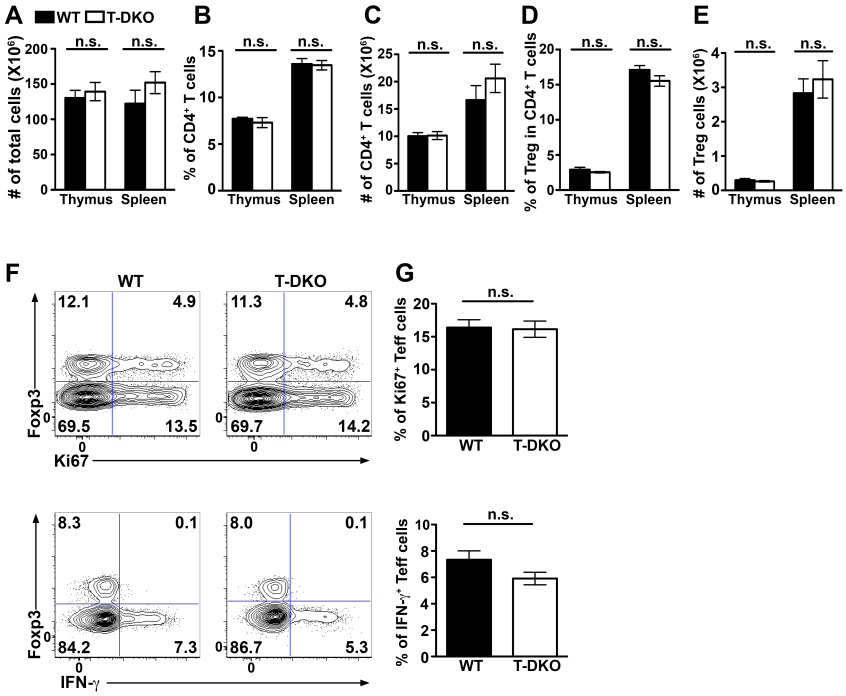

Figure 1.8: **No detectable immune phenotypes in mice with T cell-specific deletion of both miR-23 clusters at steady state.** (A-E) Cellularity of the thymus and spleen and the proportions and absolute numbers of thymic and splenic total $CD4^+$ T cells and $Foxp3^+CD4^+$ T reg cells in T-DKO and control littermates are shown. FACS analysis of $Ki67^+$ (F) and IFNγ-secreting (G) cells in splenic $CD4^+$ T cells isolated from indicated mice are shown. Data are representative of four independent experiments. n = 10.

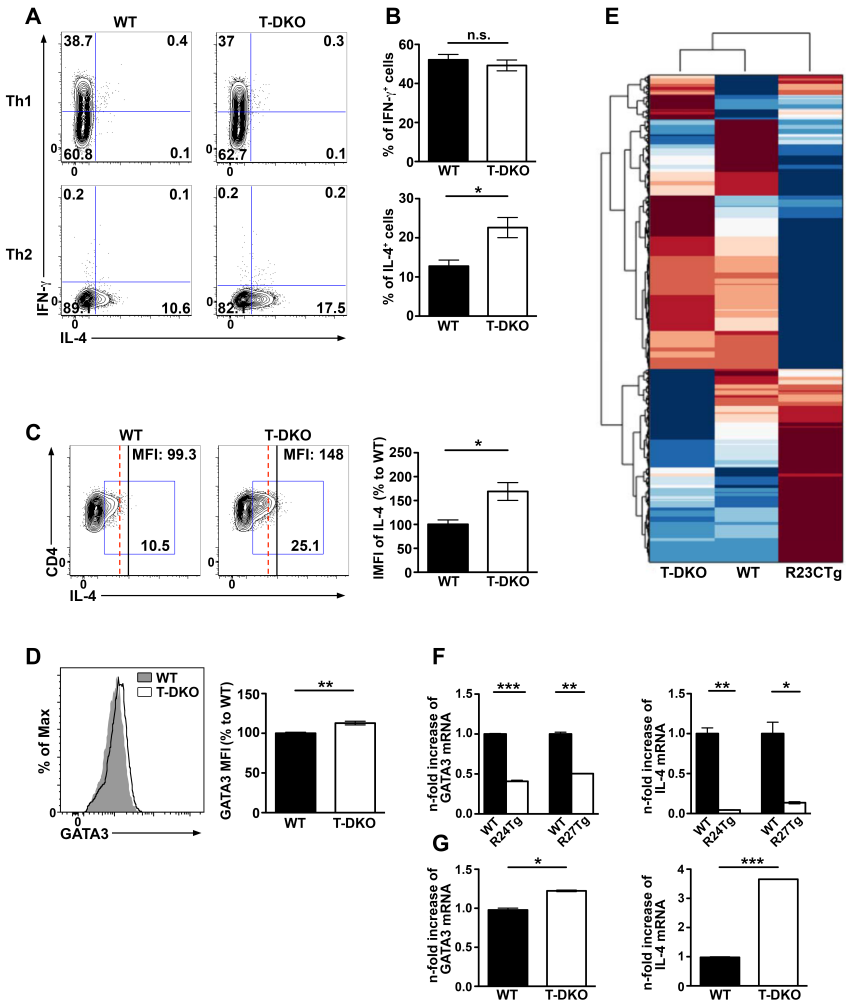

Figure 1.9: **Deletion of both miR-23 clusters in T cells resulted in increased Th2 differentiation and IL-4 expression.** (A) Clustering of RNA-seq results from T-DKO, R23CTg, and WT naive CD4$^+$ T cells based on total gene expression. FACS analysis (B), frequencies (C), and MFI of IL-4 in CD4$^+$ T cells isolated from T-DKO mice or WT littermates cultured under Th2-polarizing conditions. (D) FACS analysis and MFI of GATA3 from T-DKO CD4$^+$ T cells compared with WT CD4$^+$ T cells. qPCR analysis of IL-4 and GATA3 expression in T cells isolated from T-DKO mice (E) and R24Tg or R27Tg mice (F) upon Th2 polarization. All data are representative of two independent experiments. n = 3-6. *, P <0.05; **, P <0.01; ***, P <0.001.

Next, we sought to test whether mice harboring miR-23 cluster-deficient T cells would exhibit dysregulated Th2 immunity *in vivo* when challenged to mount a Th2 response. To this end, we first employed a model of ovalbumin (OVA)-induced airway allergic asthma in which the role of Th2 cells in driving lung inflammation, airway hyper-responsiveness, and obstruction has been well established [129]. As shown in Figure 1.10A, we could detect increased expression of every member of the miR-23 family in T cells upon OVA challenges. These results suggested that miR-23 family-mediated gene regulation in T cells might serves as a mechanism of negative feedback regulation of Th2 responses during allergic reaction. Indeed, one day after the last OVA challenge, compared to WT littermates, T-DKO mice exhibited significantly higher pulmonary resistance (Figure 1.10B). Selective increases in the percentage and the absolute number of eosinophils in bronchoalveolar lavage (BAL) fluid were also easily detected (Figure 1.10C, D). Histopathological analyses showed much greater goblet cell hyperplasia in the bronchioles and more severe eosinophilia in the perivascular and peribronchial regions of lungs from T-DKO mice than from WT controls (Figure 1.10E, F and data not shown). Exacerbated lung pathology was accompanied by further elevated production of serum total and OVA-specific IgE, a major feature of allergic asthma, in sensitized T-DKO mice (Figure1.10G). It should be noted that the observed phenotypes in T-DKO mice were not due to unrestrained T cell activation. In fact, lung-infiltrating T cells exhibited diminished proliferation and reduced activation in OVA-challenged T-DKO mice compared to those in control animals (Figure 1.10H), complementing our previous observations of enhanced proliferation and activation of T cells with miR-23 cluster overexpression. Though fewer cells were activated, the lung-infiltrating T cells yet produced increased IL-4 and reduced IFNγ (Figure 1.10I), which accords with our *in vitro* polarization studies. Similarly, higher IL-4 and lower IFNγ expression levels were also detected in lung tissues harvested from OVA-challenged T-DKO mice (Figure 1.10J). Finally, similar to our findings in mice with OVA challenges, we could also detect

elevated total IgE levels in the serum and increased eosinophils in BAL fluid when T-DKO mice were challenged with a real allergen, house dust mite (HDM) (Figure 1.10K, L). There were also more (albeit not statistically significant) IL-4 in lung tissues of T-DKO mice (Figure 1.10M). On the other hand, unlike the OVA model, we did not detect any decrease in IFNγ production in T-DKO mice likely due to the complex nature of whole HDM extracts compared to a single purified protein used in the OVA study. Altogether, these results confirmed that the miR-23 clusters in T cells play a key role in restraining Th2 responses and associated immune pathology particularly in the setting of allergic airway inflammation.

1.4 Discussion

There is little doubt that miRNAs function as important gene regulators in controlling almost every aspect of biology. However, because many miRNAs can be deleted without resulting in any apparent phenotype, there is also a growing consensus that most miRNAs do not play an indispensable role in dictating the outcomes of biological responses [87]. Nonetheless there are exceptions to the current idea of miRNAs as mere fine-tuners of biology, particularly among those miRNAs that target genes involved in positive feedback regulatory circuits. Self-reinforcing loops can amplify even the subtlest changes in gene expression introduced by miRNAs, leading to greater physiological consequences. Moreover, miRNAs can increase their biological impact through binding multiple sites on a gene target or repressing a set of genes that are in a shared pathway or protein complex [27]. The aforementioned scenarios can be further exploited by the existence of evolutionary conserved miRNA clusters where members of a given miRNA cluster often target the same gene or different components of a common biological process [67]. Additionally, duplication of such miRNA clusters in paralogs ensures rigid control of their targets. In this present study, we have provided direct genetic evidence to demonstrate how miR-23 clusters and paralogs

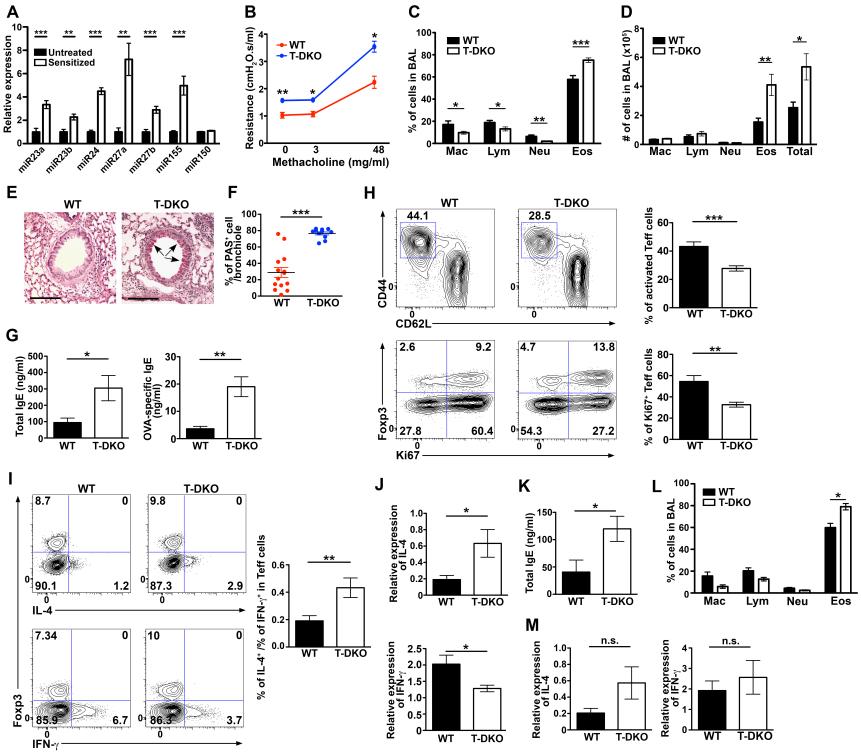

Figure 1.10: **Exacerbated eosinophilic airway inflammation in mice with T cell-specific ablation of both miR-23 clusters.** (A) qPCR analysis of different miRNA expression in lung-infiltrating T cells harvested from OVA-challenged mice. miR-155, but not miR-150, has been shown to be up-regulated during OVA challenges and were used as positive and negative controls, respectively [83]. (B) Pulmonary function test was determined 24 h after last challenge in response to increasing doses of methacholine (0, 3, and 48 mg/ml). Airway resistance of T-DKO mice and WT control littermates. (C) Frequencies and (D) absolute cell numbers of different cell populations in BAL. Mac, macrophages; Lym, lymphocytes; Neu, neutrophils; Eos, eosinophils. (E) PAS staining of lung sections from sensitized control and T-DKO mice are shown (bar, 200 um), and (F) percentages of PAS^+ mucus-secreting cells in per bronchiole were quantified. (G) Serum levels of total IgE and OVA-specific IgE levels were determined by ELISA. (H) FACS analysis and frequencies of $CD44^{hi}CD62L^{lo}$ activated T cells and $Ki67^+$ T cells from lung in OVA-challenged T-DKO mice and WT control littermates are shown. (I) Frequencies and the ratios of IL-4 and IFNγ secreting cells in lung-infiltrating $CD4^+$ T cells isolated from indicated mice were shown. (J) qPCR analysis of IL-4 and IFNγ mRNA expression in lung tissues harvested from OVA-challenged T-DKO mice or control littermates. Data are represented of three independent experiments. n = 10-14. 24 d after HDM intranasal challenges, serum levels of total IgE levels were determined by ELISA (K) and frequencies cell numbers of different cell populations in BAL were shown (L). (M) qPCR analysis of IL-4 and IFNγ mRNA expression in lung tissues harvested from HDM-challenged T-DKO mice or control littermates. Data are representative of two independent experiments. n = 8-10 *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

can exert their regulatory effects on important biology particularly in controlling T cell immunity. Specifically, we have shown that miR-24 and miR-27 collaboratively control Th2 immunity through targeting IL-4 and GATA3, respectively.

Interestingly, while HITS-CLIP analysis revealed putative binding of both miRNAs to the 3' UTR of GATA3, only miR-27 but not miR-24 seems to be functionally active in directly repressing GATA3. To this end, as IL-4 itself is a direct target of miR-24, exaggerated miR-24 regulation likely contributes to impaired IL-4 production and a further disruption of GATA3-dependent Th2 cell differentiation program considering the role of autocrine IL-4 signaling in potentiating GATA3 expression [111]. However, it should be noted that the miR-24 binding site in mice is not conserved in humans, raising the question regarding the biological relevance of miR-24-mediated IL-4 repression beyond mouse studies. Nevertheless, miR-24 has been previously shown to be able to control multiple genes with no canonical target seed sequences [63]. Similar observations in "seedless" genes targeted by miR-146a and miR-155 were also previously reported [75, 77]. It was shown that the complimentary sequences outside the seed region could contribute to the noncanonical miRNA-mediated repression albeit at relatively moderate levels compared to the canonical targeting [75]. The fact that the 3' end of miR-24 could interact with more conserved sequences in the IL-4 coding region as this putative binding site is located just one nucleotide after the stop codon of mouse IL-4 gene suggested that it might be possible that miR-24 could also control human IL-4 expression even in the absence of canonical seed sequences. Our findings of increased repression in the luciferase reporter assay with the construct containing the extended sequences from the IL-4 coding region where miR-24 could interact further supported this scenario. Alternatively, miR-24 could negatively impact human IL-4 expression in an indirect manner similar to what was reported in miR-27-mediated GATA3 regulation in humans through targeting Bmi1, a molecule that stabilizes GATA3 by blocking its proteasome-dependent degradation [36]. Indeed, our RNA-seq study revealed that many

Th2-associated genes were already downregulated in naive T cells upon overexpression of miR-24 or miR-27. Although it is uncertain as to how many of them were directly controlled by miR-24 or miR-27 since these two miRNAs predominantly repress their respective targets related to Th2 responses at the protein rather than the mRNA level, nevertheless, our results demonstrated that the miR-23 clusters, particularly miR-24 and miR-27, are able to efficiently restrain Th2 differentiation and effector function through directly and indirectly targeting multiple components within the Th2-associated gene network.

Such coordinated repression by miR-23 clusters is not likely to be limited to Th2 regulation. Given that almost every facet of T cell immunity is controlled by this miRNA family, one would imagine that a similar regulatory mechanism could be employed by the miR-23 clusters to confer proper immune function to other T cell lineages. For example, we have shown that both miR-23 and miR-27 limit the differentiation of Th17 and iTreg cell populations. As TGFβ signaling plays an integral part in the induction of both T cell lineages, it is probably not a coincidence that several of key molecules downstream of TGFβ signaling have been shown to be repressed by these two miRNAs [104]. On the other hand, we have found that contrary to miR-23 and miR-27, miR-24 promoted iTreg and Th17 responses. This surprising finding was interesting but not completely unexpected as HITS-CLIP analysis has identified miR-24 as the only miR-23 family member that could target Smad7, a negative regulator of TGFβ signaling, suggesting that miR-24 could exert an effect on iTreg and Th17 cells opposite to the other cluster members through augmenting TGFβ signaling [75]. Nevertheless, the fact that different members of the same miRNA cluster can antagonize each other in controlling a specific biological process seems to be counterintuitive and raises a question as to how such an unproductive feature in miRNA-mediated gene regulation could be retained evolutionarily. One possibility is that in some situations, one member of the miRNA cluster could exert an opposite function to fine tune the regulatory effects of the entire miRNA family. Another probable clue for this puzzle was provided by a previous

report in which Bmp2, a member of TGFβ superfamily, was shown to promote adipocytic differentiation from mesenchymal stem cells by specifically upregulating miR-24 but not miR-23 or miR-27 [120]. It is intriguing to speculate that while in most cases, individual members of miR-23 family would function cooperatively to maximize their impact, under certain circumstances, a given miR-23 family member could be differentially expressed to control a specific type of T cell response. Finally, beyond regulating T cell immunity, it has been previously documented that miR-23a cluster promotes the differentiation of myeloid cells from hematopoietic progenitors while inhibiting B cell development [58]. In dendritic cells, upregulation of either miR-23b or miR-27a was shown to confer tolerogenic activities, allowing them to induce more Treg cells [86,143]. Future studies will continue to unravel the role of this miRNA family not only in T cell immunity but also in regulating other aspects of the immune system.

1.5 Materials and Methods

Mice and experimental design.

Foxp3^{GFP} [31] and Foxp3^{GFPKO} mice [32] were described previously. The targeting constructs for miR-23bFB^{fl}, miR-23aC^{fl} and miR-23bC^{fl} were generated using recombineering. For detailed information see the National Cancer Institute recombineering website (http://redrecombineering.ncifcrf.gov/). Mice on a B6 genetic background with the germ-line transmission were bred to Flp deleter mice to remove the neomycin resistance cassette. T cell- specific deletion of both miR23 clusters were achieved by breeding miR23a/bC$^{fl/fl}$ mice to CD4-cre mice, respectively. The targeting constructs for R23CTg, R23Tg, R24Tg and R27Tg were generated similar to what was described previously [136]. Mice with T cell-specific overexpression of the entire miR-23 family and individual members were obtained by breeding the aforementioned mice to CD4-cre mice. All mice were maintained and handled

in accordance with the Institutional Animal Care and Use Guidelines of UCSD and NIH Guidelines for the Care and Use of Laboratory Animals and the ARRIVE guidelines.

miRNA Expression Profiling and Quantitative PCR Analysis

Foxp3^{GFP-} Treg cells and naive CD62L^{hi} Foxp3^{GFP-} Tconv cells from Foxp3^{GFP-} mice were sorted on FACSAria (Becton Dickinson) followed by one day culture with 100U/ml IL-2 with or without plate-bound 1 ug/ml αCD3 and/or 1 ug/ml αCD28. Isolated RNA from harvested cells (duplicates of each sample) was subject to miRNA expression profiling analysis using miRCURY LNA^{LM} microRNA Arrays (Exiqon). For confirming the expression levels of miR-23 clusters by qPCR, Taqman "stem-loop" real-time RT-PCR was performed as demonstrated previously [77].

For detecting cytokine levels in sensitized lungs, total RNA of lung was extracted by using miRNeasy kit (QIAGEN), cDNAs were generated by iScript cDNA synthesis kit (Bio-Rad), and real-time PCR was performed using SYBR Green PCR kits (Applied Biosystems). Primers are as follows: IL-4, 5'- TTGAACGAGGTCACAGGAGA-3' (F) and 5'-AAATATGCGAAGCACCTTGG-3' (R); IFNγ, 5'- GCGTCATTGAATCACACCTG-3' (F) and 5'- GAGCTCATTGAATGCTTGGC-3' (R); Gapdh, 5'- CGTCCCGTAGACAAAATGGT-3' (F) and 5'- TCAATGAAGGGGTCGTTGAT-3' (R).

Ex Vivo Phenotyping and Flow Cytometry

Single cell suspensions of thymus and spleen were prepared by slide mechanical grind. To isolate lymphocytes in lung and lamina propria, tissues were cut and washed in plain RPMI1640, and epithelial cells were removed (5mM EDTA and 1mM DTT, lamina propria only), followed by enzymatic digestion (0.16U/ml Liberase TL, Roche) and centrifugation with 47% of Percoll gradient to enrich lymphocytes. Cell were stained in FACS buffer (5% FBS in PBS) containing Fixable Viability Dye eFluor 780 or 450 (eBioscience) with the

following antibodies for surface staining: CD4, CD8, CD44 and CD62L (all eBioscience). To detect cytokine production, cells were stimulated in a 96 well plate with PMA (50 ng/ml), ionomycin (0.5 ul/ml) and Brefeldin A (1 ug/ml, all Sigma-Aldrich) solution for 4hr at 37C before staining. Intracellular staining of Ki67, Foxp3, IL-4, IL-17A and IFNγ was performed following fixation and permeabilization according to manufacturer's instructions. Data were analyzed with FlowJo software (TreeStar).

In Vitro Th Differentiation

$CD62L^{+}CD25^{-}$ naive T cells in spleen were sorted by FACSAria flow cytometers (BD Biosciences) from 6-8 weeks old mice, and were stimulated for 4 days with 1ug/ml αCD3, and mitomycin C (Sigma-Aldrich)-treated APC and 50 U/ml recombinant human IL-2 under Th1-, Th2-, iTreg or Th17-polarizing conditions. Th cell polarization mediums were supplemented as follows: for Th1 differentiation, 2 U/ml IL-12 and 10 ug/ml αIL-4; for Th2 differentiation, 20 ng/ml IL-4 (in T-DKO study, 10 ng/ml was used), 10 ug/ml αIL-12 and 10 ug/ml αIFNγ; for iTreg differentiation, 1 ng/ml human TGFβ; for Th17 differentiation, 2 ng/ml hTGFβ, 20 ng/ml IL-6. All cytokines were obtained from Peprotech and all blocking Abs were obtained from Bio-XCell. Surface and intracellular cytokines were stained and analyzed as previously described.

In Vitro Suppression Assay

FACS purified 4 x 10^4 naive $CD4^{+}CD25\text{-}CD62L^{hi}$ T cells and $CD4^{+}CD25^{hi}$ Treg cells isolated from R23CTg mice or WT control littermates were mixed at the indicated ratios and stimulated with 1ug/ml αCD3 antibody in the presence of irradiated (2000 rads) splenocytes. T cell proliferation was assessed by with 3H-TdR incorporation (cpm) in triplicate cultures during the last 8 hr of culture.

Luciferase Reporter Assay

The 3'UTR region of GATA3 or IL-4 as well as the 3'UTR plus partial sequences in the coding region of IL-4 were cloned into psiCheck2 (Promega). miR-23a, miR-24, miR-27a sequences were respectively cloned into pMDH-PGK-EGFP similar to what was described previously [79]. To generate GATA3 or IL-4 3'UTR mutants, site-directed mutagenesis was performed (Agilent technologies). One day before transfection, HEK293T cells were plated at 6.5 x 10^4 cells per well on a 24-well plate. PsiCheck2 bearing WT 3'UTR or corresponding mutant 3'UTR were transfected to HEK293T cells with control vector or miRNA expressing plasmid using Fugene 6 (Promega). Luciferase activities were assessed at 24hr post transfection using Dual-Luciferase Reporter assay system (Promega) according to manufacturer's protocol

Immunoblotting

Naive $CD4^+$ T cells (1 x 10^6 cells/ml) were cultured in the 24-well plate coated with 2ug/ml of αCD3 and αCD28 under the Th2 polarizing condition. Cells were harvested 4 days later and subjected to lysis with RIPA buffer supplemented with 1mM of PMSF for 20 min. Cell lysates were separated by SDS-PAGE and transferred to PVDF membrane (BIO-RAD). Antibodies against GATA3 (Bio-Legend) and β-actin (Sigma) were used to visualize the corresponding proteins. The proteins were quantified with Image J.

Retrovirus Transduction

pMIG-mGATA3 (Addgene plasmid 21629) [59], a gift from Zena Werb (Harvard, Boston, MA) were used as a template to subclone into pMIR-RI. To generate retrovirus, pMIR-R1 or pMIR-R1-GATA3 (without 3'UTR) was transfected with pCL-Eco into HEK293T cell using Fugene 6 (Promega). Retroviral supernatant were collected at 48hr after transfection. Naive $CD4^+$ T cells (1 x 10^6 cells/ml) were stimulated with 2ug/ml of

αCD3 and 2ug/ml of αCD28 for 24hr followed by spin infection for 90 min at 2,000 rpm in the presence of 8ug/ml of polybrene (Millipore). Cells were cultured in the Th2 polarizing condition and harvested at 4 days after retrovirus transduction.

Gene Expression Profiling Analysis

$CD25^{-}CD44^{lo}CD62L^{hi}$ naive $CD4^{+}$ T cells were FACS sorted from 6 weeks WT, R23Tg, R24Tg, R27Tg, R23CTg and T-DKO mice and poly-A RNA-sequencing was performed using 3 biological replicates for each cell population. Approximately 30 million uniquely alignable reads per sample reads per sample were generated. Reads were aligned to the mouse genome (mm9, NCBI37) using STAR [25]. RNA-seq experiments were normalized and gene expression values were generating for RefSeq annotated transcripts using HOMER [42]. Gene expression clustering was performed using Cluster 3.0 and visualized using Java TreeView. For the cumulative distribution function (CDF) plots, target sites were restricted to perfect seed complementarity between positions 2 and 7 of the corresponding miRNA with positive Argonaute binding peaks in the HITS-CLIP dataset [75]. Empirical cumulative distributions were computed using Matlab (R2014b) to display the log2(miRNA Tg/WT) against the cumulative frequency of Th2-associated [119] and GO annotation: http://geneontology.org/) or all genes. String Analysis (http://string-db.org/) was performed to identify potential physical and/or functional interactions between Th2 genes that were proven miR-24 or miR-27 targets or downregulated by miR-24 and/or miR-27 as shown from the RNA-seq results. The interaction map was reconstructed manually with additional information obtained from the HITS-CLP data analysis. RNA-seq data are available from NCBI under accession number GSE75909.

In Vivo Allergic Airway Inflammation Models

Mice were sensitized by two intraperitoneal injections of 50ug of ovalbumin (OVA, Worthington) that had been emulsified with 0.8 mg of aluminum hydroxide (Pierce) in 200 ul PBS on days 0 and 12. Mice were then challenged on days 24, 26, and 28 by intra-nasal injection of 20 ug (or 10 ug as low-dose group) of OVA. One day after the last OVA challenge, mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) *via* intraperitoneal injection. Airway reactivity to methacholine was measured in intubated and ventilated mice (flexiVent ventilator; Scireq). Mice were exposed to nebulized PBS and subsequently to increasing concentrations (3 and 48 mg/ml) of nebulized methacholine (Sigma-Aldrich) in PBS. The dynamic airway resistance was determined by using Scireq software. After pulmonary function test, the lungs of the mice were lavaged with 0.7 ml of normal saline. The BAL cells were centrifuged onto slides at 1,000 rpm for 3 minutes in a Shandon Cytospin (Thermo). The cells were then stained with Wright Giemsa stain (Protocol) for 3 minutes. Two hundred cells per slide were counted under a microscope, and those with different morphology were noted. In some experiments, HDM extract (Dermatophagoides pteronyssinus, Greer labs, cat XPB82D3A2.5) instead of OVA was used. Briefly, mice were exposed intranasally to 100 ug in 50 ul PBS on days 0, 7, 14 and 21 and were sacrificed on day 24 for further assessment similar to the work described in the OVA study.

Histology

To assess immunopathology, different tissues were removed and immediately fixed in 10% formalin solution for hematoxylin and eosin (H&E) staining of sections embedded in paraffin. Inflammation was examined and scored blindly by a UCSD pathologist. As for additional assessment of lung pathology during airway allergic reaction, periodic acid-Schiff (PAS) stain was performed to detect goblet cell hyperplasia. To quantitate the level of

goblet cell hyperplasia in the airway, the percentage of PAS^+ epithelial cells in individual bronchioles was counted, and at least 10 bronchioles were determined.

ELISA

The concentrations of total and OVA-specific IgE in serum were evaluated with ELISA kits (BioLegend) according to the manufacturer's instructions.

Statistical Analyses

Unpaired Student's t test was performed using Prism software (GraphPad). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ in all data.

1.6 Acknowledgements

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Rajewsky, Alexander Y. Rudensky, Li-Fan Lu he Journal of Experimental Medicine (2016). *The Journal of Experimental Medicine* (2016) * denotes co-first authorship. The author of this dissertation was the fourth investigator and author of this paper.

Chapter 2

Excessive miR-27 expression impairs regulatory T cell-mediated immunological tolerance

2.1 Abstract

MicroRNAs (miRNAs) are tightly regulated in the immune system as aberrant expression of miRNAs often results in hematopoietic malignancies and autoimmune diseases. Previously, elevated levels of miR-27 in T cells isolated from multiple sclerosis patients has been suggested to facilitate disease progression through inhibiting Th2 immunity and promoting pathogenic Th1 responses. Here we demonstrate that while mice with T cell-specific overexpression of miR-27 harbor dysregulated Th1 responses and develop autoimmune pathology, these disease phenotypes are not driven by miR-27 in effector T cells in a cell-autonomous manner but rather result from a perturbed regulatory T (Treg) cell compartment. Excessive miR-27 expression in T cells severely impairs Treg cell differentiation. Moreover, Treg cells with exaggerated miR-27-mediated gene regulation exhibit diminished homeostasis

and suppressor function *in vivo*. Mechanistically, miR-27 represses several known as well as previously uncharacterized targets that play critical roles in controlling multiple aspects of Treg cell biology. Collectively, our data show miR-27 functions as a key regulator in Treg cell development and function and suggest that proper regulation of miR-27 is pivotal to safeguard Treg cell-mediated immunological tolerance.

2.2 Introduction

MicroRNA (miRNA)-mediated gene regulation plays crucial roles in the development and function of the immune system [64, 94]. The onset of many human hematopoietic malignancies can be directly attributed to the deletion or amplification of genomic regions where miRNAs are encoded [11, 118, 121]. Similarly, abnormal expression of miRNAs in immune cells was also found to be highly correlated with the pathogenesis of a wide range of autoimmune disorders [64, 115]. Accumulating experimental evidence has further demonstrated that certain miRNAs promote disease progression whereas others function as negative regulators to limit immune activation and resultant pathologies [7, 36, 92, 101, 137]. The identification of such a causative role of miRNAs in autoimmune disease pathogenesis suggests miRNAs can serve as biomarkers and potential therapeutic targets for treating immunological disorders. Nevertheless, the precise molecular and cellular mechanisms by which miRNAs regulate autoimmunity requires to be further investigated before making miRNA-based immunotherapy a possibility.

Previously, miR-27, a member of the miR-23˜27˜24 family, was found to be highly up-regulated in T cells isolated from patients with multiple sclerosis (MS) [36]. It was shown that through repressing Bmi1, a molecule that stabilizes Gata3, miR-27 inhibits Th2 differentiation and promotes proinflammatory Th1 autoimmune responses. Consistent with this study, we have also recently found that Gata3 itself can be directly targeted by miR-27,

substantiating the idea that miR-27 as a negative regulator of Th2 immunity [18]. Moreover, our recent finding of elevated IFNγ responses and lympho-hyperactivated phenotypes in mice harboring T cells with miR-27 overexpression further supported the conclusion drawn by the aforementioned study in human MS patients [18, 36]. Surprisingly, the role of miR-27 in driving IFNγ-mediated Th1 autoimmune responses implicated by these two reports seems to be at odds with previous studies where miR-27 was shown to be a potent repressor of IFNγ and T cell receptor (TCR)-mediated activation [13, 38]. The fact that overexpression of miR-27 in T cells resulted in impaired Th1 differentiation further suggested that miR-27-mediated gene regulation limits rather than promotes Th1 responses [18]. Such contradictory results warrant a more careful examination of the exact role of miR-27 in controlling T cell immunity.

In this current study, we show that miR-27 plays a cell-intrinsic role in negatively regulating effector T (Teff) cell homeostasis, activation and cytokine production. Our results suggest that the dysregulated T cell responses and autoimmune phenotypes in mice with T cell-specific miR-27 overexpression are on the other hand likely due to a perturbed regulatory T (Treg) cell compartment. Global transcriptom study combined with high-throughput sequencing of RNAs isolated by cross-linking immunoprecipitation (HITS-CLIP) analysis revealed that many genes previously associated with Treg cell differentiation and suppressor function are directly targeted by miR-27. In particular, we found that forced expression of miR-27 during thymocyte development severely impaired Treg cell generation by repressing c-Rel, a member of NFκB transcription factors known for its indispensable role in initiating Foxp3 transcription [45, 76, 106, 144]. Exaggerated miR-27-mediated regulation of other Treg suppressor molecules such as IL-10 and Granzyme B (GZMB) further contributes to the functional defects in peripheral Treg cell populations as miR-27 overexpressing Treg cells failed to prevent autoimmune pathologies in the adoptive transfer colitis model as well as in mice with normal thymic Treg cell development. Together, our data provides new cellular

and molecular insights into miR-27-driven T cell autoimmunity and suggests that optimal expression of miR-27 is crucial to maintain immunological tolerance through regulating multiple aspects of Treg cell biology

2.3 Results

Mice harboring T cells with miR-27 overexpression exhibited dysregulated IFNγ responses despite diminished IFNγ production by T cells on a per-cell basis

As previously reported, T cells in peripheral blood mononuclear cells (PBMCs) isolated from human MS patients exhibited very high levels (up to ˜30 folds induction) of miR-27 compared to those from healthy donors [36]. To determine the precise role of miR-27 in regulating IFNγ-dependent Th1 responses, we sought to further characterize mice harboring T cells that express miR-27 to elevated levels similar to T cells from human MS patients (CD4-cre R27Tg) [18]. Previously, we showed that the spontaneous lympho-hyperactivation diseases which developed in mice with T cell-specific overexpression of the miR-23˜27˜24 family (CD4-cre R23cTg) could be mostly recapitulated when miR-27 alone was overexpressed [18]. Further analysis of mice of different ages revealed that the T cell activation phenotypes could already be observed in ˜3-month-old CD4-cre R27Tg mice (Fig. 2.1A). Five months after birth, these mice started to develop autoimmune lesions manifested in runting, failure to thrive, lymphadenopathy and splenomegaly, and lymphocytic tissue infiltration that was particularly severe in the lung, colon, and stomach (Fig. 2.1B and data not shown). Interestingly, regardless the absence or presence of increased IFNγ$^+$ T cell frequencies in young or older CD4-cre R27Tg mice, respectively (Figure 2.2A and B), we could consistently detect reduced IFNγ production by miR-27-overexpressing T cells on a per-cell basis (Figure 2.2C), supporting a previous notion that miR-27 functions a potent

repressor of IFNγ in T cells [13, 38].

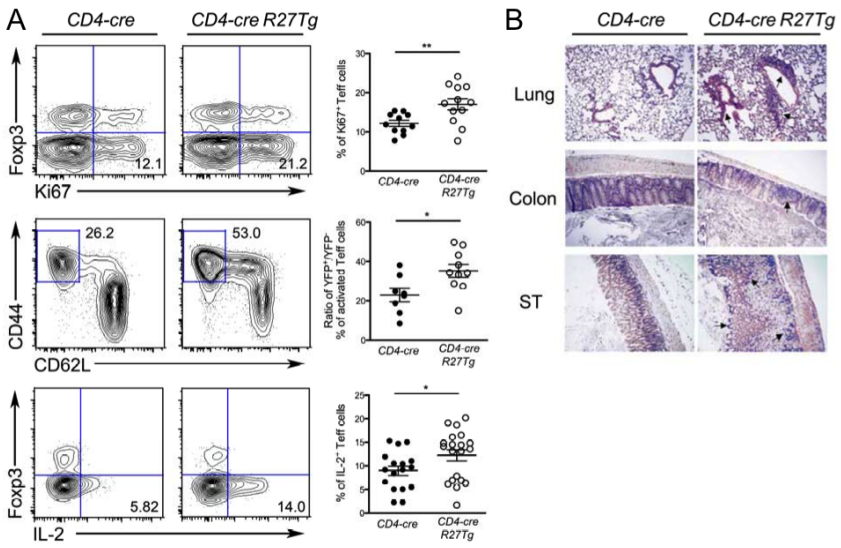

Figure 2.1: **CD4-cre R27Tg mice exhibited autoimmune phenotypes.** (A) FACS analysis and frequencies of Ki67^{+}, CD44^{hi} CD62L^{lo} subset and IFNγ^{+} cells in Foxp3^{-}CD4^{+} Tconv cells in spleen from CD4-cre R27Tg mice (>14 wks) and control littermates were shown. (B) H and E-stained sections of the lung, colon, and stomach (ST) from the indicated mice. Data are representative of four independent experiments. Each symbol represents an individual mouse, and the bar represents the mean. *p <0.05, **p <0.01

Enforced expression of miR-27 in T cells results in reduced Treg cell numbers

The observation of dysregulated IFNγ responses in CD4-cre R27Tg mice that harbored T cells with diminished capacity to produce IFNγ raised a question as to whether or not the autoimmune pathology developed from a defect in the Treg cell compartment. After all, miR-27 was also overexpressed in Treg cells in mice in which the miR-27 transgene is expressed in all T cells. To examine this possibility, we analyzed CD4-cre R27Tg mice of young age (<3 months) to avoid the potential confounding effects from the aforementioned late-developing lympho-hyperactivation phenotypes. Indeed, while young mice did not exhibit any clear alteration in total thymic or splenic cellularities compared to WT littermates, we could easily detect significant reduction in both thymic and splenic Treg

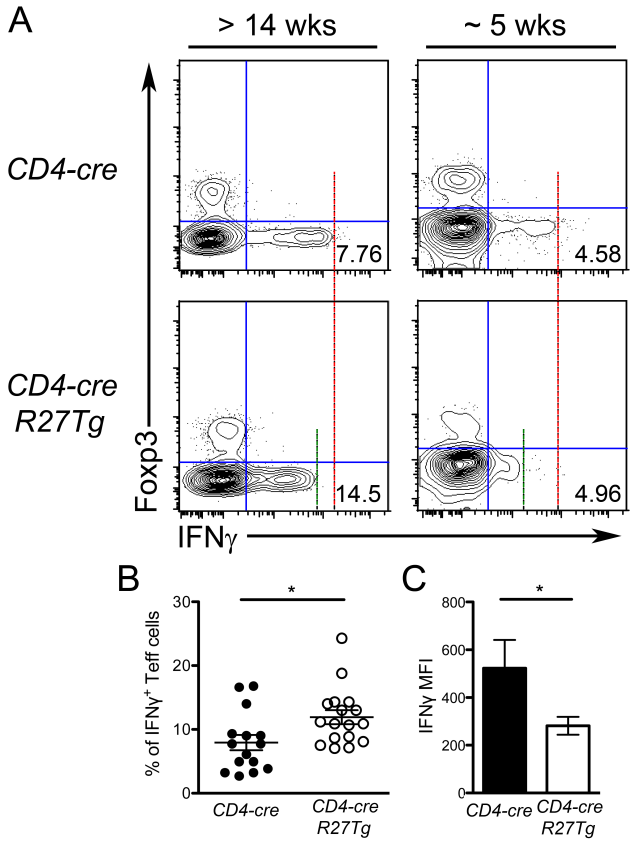

Figure 2.2: **Diminished IFNγ production by miR-27-ovexpressing T cells in CD4-cre R27Tg mice.** (A) FACS analysis of IFNγ$^+$ cells in Foxp3$^-$ CD4$^+$ Tconv cells in mice of different ages (>14 wks v.s.˜5wks). (B) Frequencies and (C) Mean fluorescence intensity (MFI) of IFNγ from R27Tg Tconv cells compared with WT Tconv cells in mice older than 14 weeks Data represent mean ± SD and are representative of three independent experiments. Each symbol represents an individual mouse, and the bar represents the mean. *p <0.05.

cell populations (Figure 2.3A-G). The observed Treg phenotype in CD4-cre R27Tg mice was mostly consistent with our previous findings in mice with T cell-specific overexpression of the entire miR-23˜27˜24 family [18], with an even greater percentage of the former showing decreased peripheral Treg cell frequencies (Figure 2.3H). In contrast, some mice with T cell-specific overexpression of miR-24 (CD4-cre R24Tg) actually harbored increased thymic Treg cells while no change in thymic and peripheral Treg cell frequencies could be detected in mice containing miR-23-overexpressing T cells (CD4-cre R23Tg) (Figure 2.3H). As overexpression of miR-27 was also previously shown to strongly impair TGFβ-mediated iTreg cell differentiation [18, 114], together these results suggested that miR-27 is the major member of the miR-23˜27˜24 family that negatively regulates the Treg cell population.

miR-27 inhibits effector T cell homeostasis and function in a cell-autonomous manner

While insufficient numbers of Treg cells likely contributed to the lympho-hyperactivation phenotypes observed in CD4-cre R27Tg mice, the possibility that overexpression of miR-27 might lead to dysregulated T cell responses in a Tconv cell-intrinsic manner still cannot be fully excluded. To directly address this issue, we performed bone marrow (BM) chimeras studies by transferring BM cells from CD4-cre R27Tg mice or WT littermates mixed with BM cells from Ly5.$^+$ B6 mice at a 1:1 ratio into irradiated Rag1-deficient recipients as described previously [78] (Figure 2.4A). Unlike CD4-cre R27Tg mice, the CD4-cre R27Tg/Ly5.1$^+$ B6 chimeric mice were healthy and showed no sign of autoimmunity and no clear alteration in different thymocyte subsets (Figure 2.5A). Nevertheless, consistent with our observation in unmanipulated CD4-cre R27Tg mice, we could easily detect marked reductions in the frequencies of R27Tg Treg cells within both the thymic and peripheral CD4$^+$ T cell populations as compared to the corresponding WT cell subsets in the control mixed chimeras (Figure 2.4B). Moreover, R27Tg Treg cells also exhibited reduced proliferative capacity as

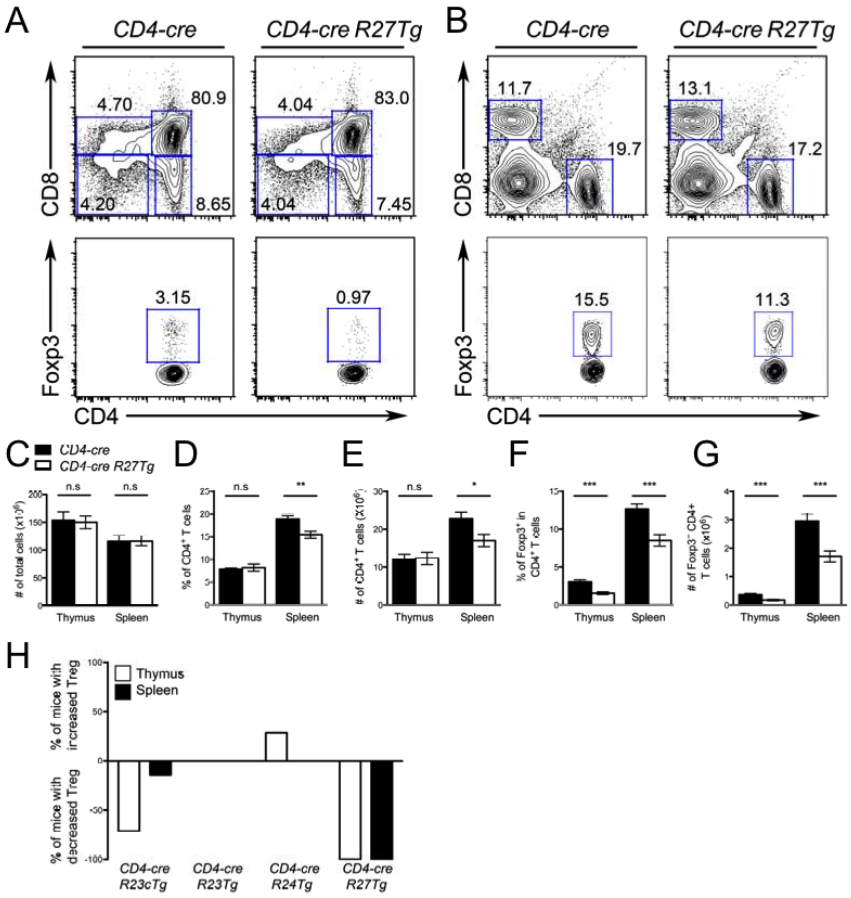

Figure 2.3: **CD4-cre R27Tg mice harbored reduced Treg cell numbers.** FACS analysis of (A) thymus and (B) spleen of 6-12 wks old CD4-cre R27Tg mice and WT littermates. (C-G) Cellularity of the thymus and spleen and the proportions and absolute numbers of thymic and splenic total $CD4^+$ T cells and $Foxp3^+CD4^+$ Treg cells in CD4-cre R27Tg mice and control littermates are shown. (H) Percentages of mice with overexpression of the whole cluster or individual members exhibited increased or decreased Treg cell frequencies in thymus and spleen compared to corresponding WT littermates were shown. Data represent mean ± SD and are representative of three independent experiments (n= 6-17). *p <0.05, **p <0.01, ***p <0.001

shown by reduced Ki67 staining (Figure 2.6), indicating a cell-intrinsic role of miR-27 in controlling Treg cell homeostasis. On the other hand, contrary to what has been shown in CD4-cre R27Tg mice, we observed reduced R27Tg $CD4^+$ T cell frequencies in the periphery with diminished proliferation, reduced activation and impaired cytokine production compared to their WT counterparts (Figure 2.4C-E and 5B). These results implied that the cell-autonomous role of miR-27 in Teff cells is to inhibit rather than promote homeostasis and effector function and strongly suggested that a perturbed Treg cell compartment in CD4-cre R27Tg mice is responsible for the observed autoimmune pathology.

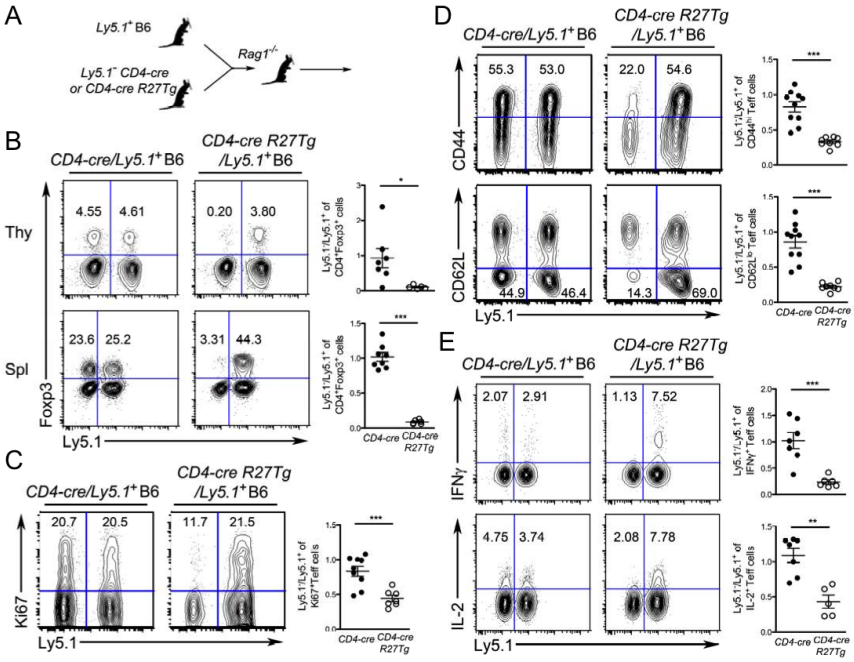

Figure 2.4: **Excessive miR-27 expression impaired Teff cell activation and function.**(A) Schematic of generation of mixed BM chimeric mice. (B) FACS analysis and ratios of frequencies of $Ly5.1^-Foxp3^+$ and $Ly5.1^+Foxp3^+$ cells within each donor-derived total $CD4^+$ T cell population from both the thymus and spleen. FACS analysis and ratios of frequencies of (C) $Ly5.1^-Ki67^+$ and $Ly5.1^+Ki67^+$ (D) $Ly5.1^-CD44^{hi}$ and $Ly5.1^+CD44^{hi}$; $Ly5.1^-CD62L^{lo}$ and $Ly5.1^+CD62L^{lo}$ as well as (E) $Ly5.1^-IFN\gamma^+$ and $Ly5.1^+IFN\gamma^+$; $Ly5.1^-IL\text{-}2^+$ and $Ly5.1^+IL\text{-}2^+$ splenic Tconv cells. FACS data represent three independent experiments. Each symbol represents an individual mouse, and the bar represents the mean. *p <0.05, **p <0.01, ***p <0.001.

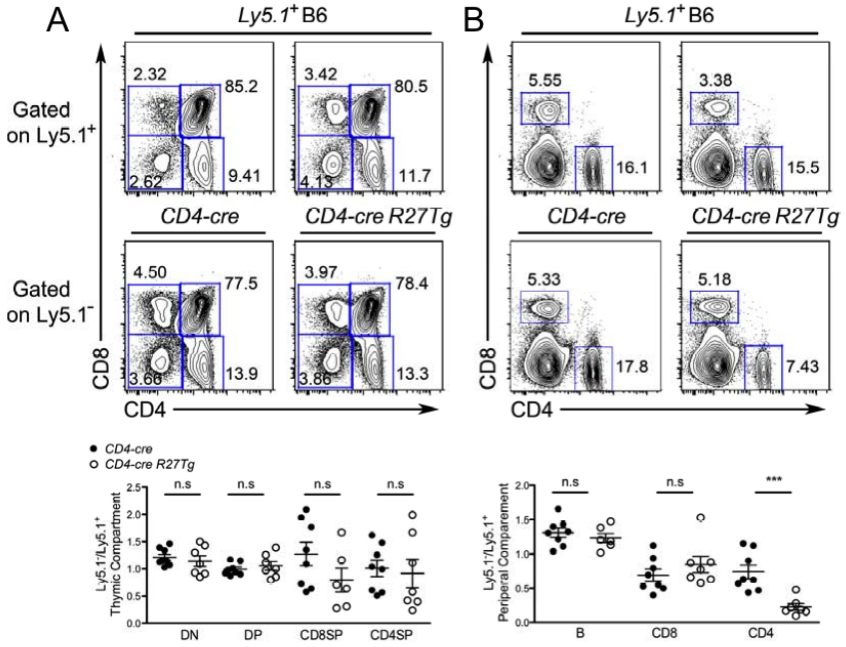

Figure 2.5: **Overexpression of miR-27 in T cells led to reduced CD4$^+$ T cell population in the periphery.** FACS analysis and ratios of frequencies of different Ly5.1$^-$ and Ly5.1$^+$ (A) thymocyte subsets or (B) splenocyte populations within each donor-derived compartment 8 wks after BM transfer. FACS data are representative of three independent experiments. Each symbol represents an individual mouse, and the bar represents the mean. ***p <0.001.

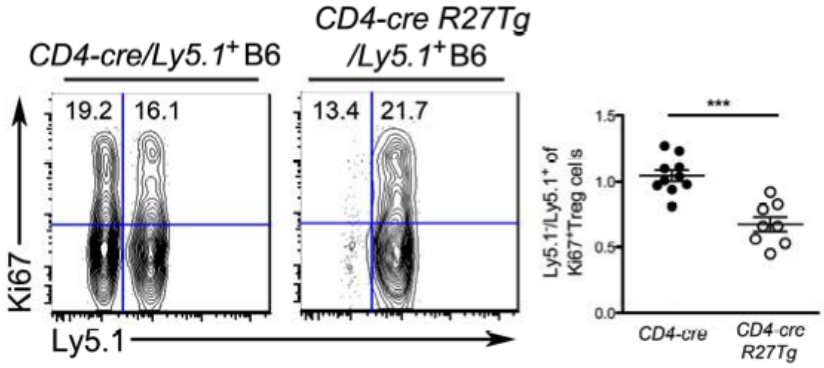

Figure 2.6: **Diminished proliferation capacity in Treg cells with excessive miR-27 expression.** FACS analysis and ratios of frequencies of (C) Ly5.1$^-$Ki67$^+$ and Ly5.1$^+$Ki67$^+$ splenic Foxp3$^+$ Treg cells. FACS data are representative of three independent experiments. Each symbol represents an individual mouse, and the bar represents the mean. ***p <0.001.

Excessive miR-27 expression impairs both thymic Treg cell development and peripheral Treg cell homeostasis

Based on our observation of reduced thymic Treg cell numbers in CD4-cre R27Tg mice, we hypothesized that the expression of miR-27 needs to be tightly regulated and downregulation of miR-27 prior to Foxp3 expression is required to ensure normal Treg cell development. Indeed, among all thymocyte populations, we have found that miR-27a (and miR-27b to a lesser degree) was expressed at the lowest level in the Foxp3- CD4SP subset (Figure 2.7A). On the other hand, the expression level of miR-24, another member of miR-23˜27˜24 family which seemed to promote rather than inhibit thymic Treg cell development upon overexpression (Figure 2.3C), did not change during the transition of DP to CD4SP cell stages (Figure 2.8). Nonetheless, it remains possible that miR-27 inhibits only Treg cell homeostasis similar to how it regulates Teff cells described above. To address this, we generated heterozygous Foxp3$^{cre/+}$ R27Tg female mice which harbor a Treg cell population with miR-27 overexpression driven by Foxp3 and a WT Treg cell population caused by X chromosome inactivation [73]. Moreover, as Cre recombinase is fused to a yellow fluorescent protein (YFP), Treg cells subjected to Cre-mediated transgene expression can be detected based on the expression of YFP [107]. Like the mixed BM chimeras, Foxp3$^{cre/+}$ R27Tg mice did not develop any detectable immune phenotype likely due to the presence of WT Treg cells. Interestingly, when the expression of miR-27 transgene was induced strictly after thymic Treg cells are differentiated (i.e. expression of Foxp3), we could only detect reduced proportions of Foxp3-driven YFP$^{cre/+}$ miR-27-overexpressing Treg cells in the spleen and peripheral tissues whereas the frequencies of thymic Treg cells were comparable regardless the miR-27 expression amounts (Figure 2.7B), suggesting that excessive miR-27 expression prior to Foxp3 induction perturbs Treg cell development in the thymus. Moreover, continued miR-27 overexpression after Foxp3 induction would further impair peripheral Treg cell homeostasis observable in the aforementioned proliferation defects and impaired

survival (Figure 2.7C). On the other hand, miR-27 seemed to only play a minimal role in controlling Treg cell tissue trafficking as the frequencies of miR-27-overexpressing Treg cells compared to their WT counterparts were similarly reduced across different tissues (Figure 2.9A). Consistent with this finding, except for lower CD103 expression, no reduction in the expression of the corresponding chemokine receptors in Treg cells with excessive miR-27 expression could be detected (Figure 2.9B and C).

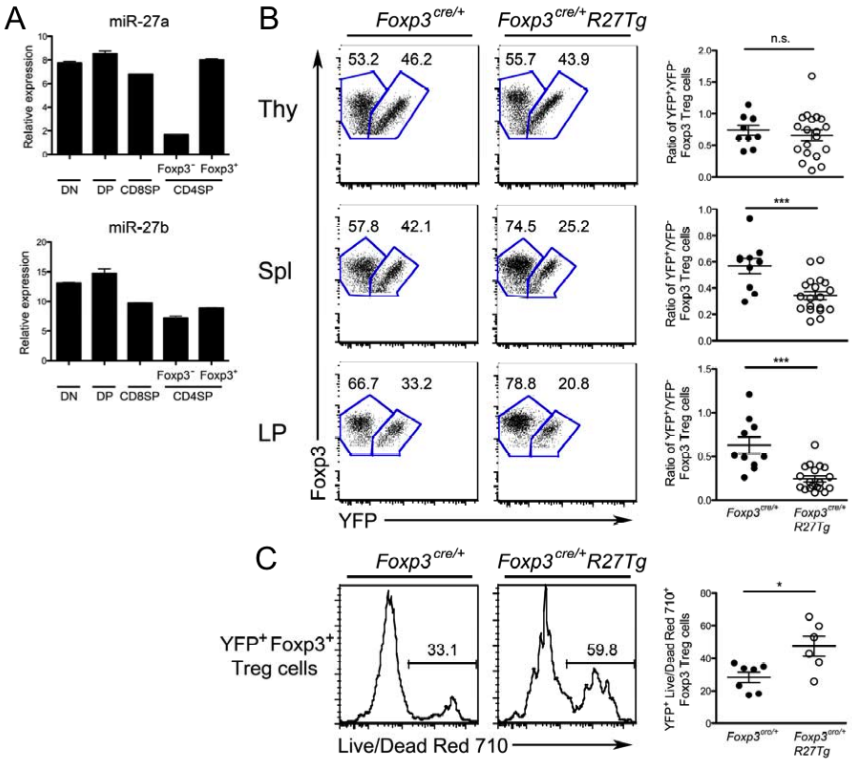

Figure 2.7: **Optimal miR-27 expression is required to maintain normal Treg cell development and homeostasis.**(A) qPCR analysis of the expression of miR-27a/b in different thymocyte subsets. Data represent mean ± SD and are representative of three independent experiments (n=3-6). (B) FACS analysis and ratios of YFP^{cre+} and YFP^{cre-} cells within $Foxp3^{+}CD4^{+}$ T cell population from $Foxp3^{cre/+}$ R27Tg mice or $Foxp3^{cre/+}$ control females. FACS data represent four independent experiments. (C) Viability of $YFP^{cre/+}$ Treg cells from indicated mice was measured by Live/Dead staining after *in vitro* stimulation with plate-bound αCD3 and αCD28 for 6 hr. FACS data represent three independent experiments. Each symbol represents an individual mouse, and the bar represents the mean. *p <0.05, ***p <0.001.

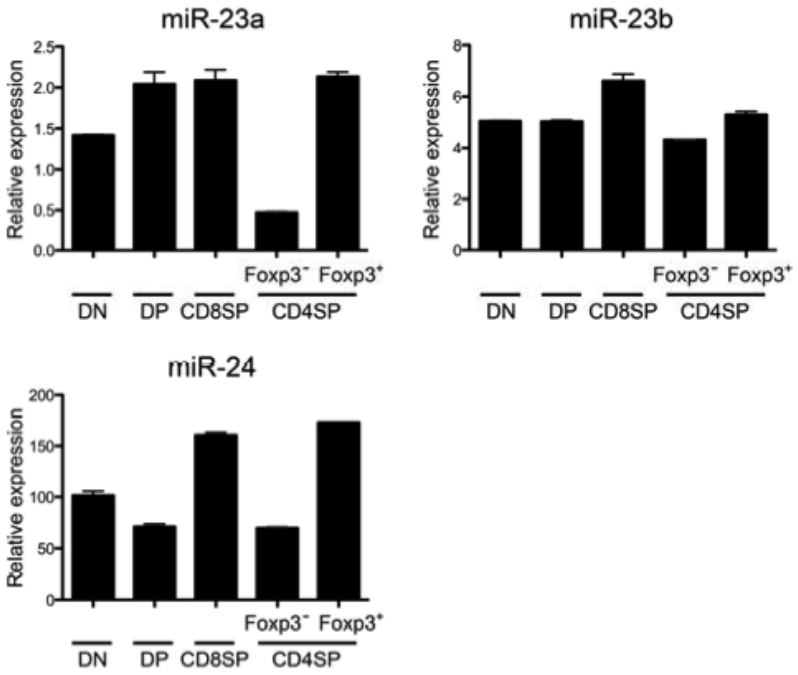

Figure 2.8: **Expression of other miR-23˜27˜24 members in different thymocyte subsets.**qPCR analysis of the expression of miR-23a/b and miR-24 in different thymocyte subsets. Data represent mean ± SD and are representative of three independent experiments (n=6).

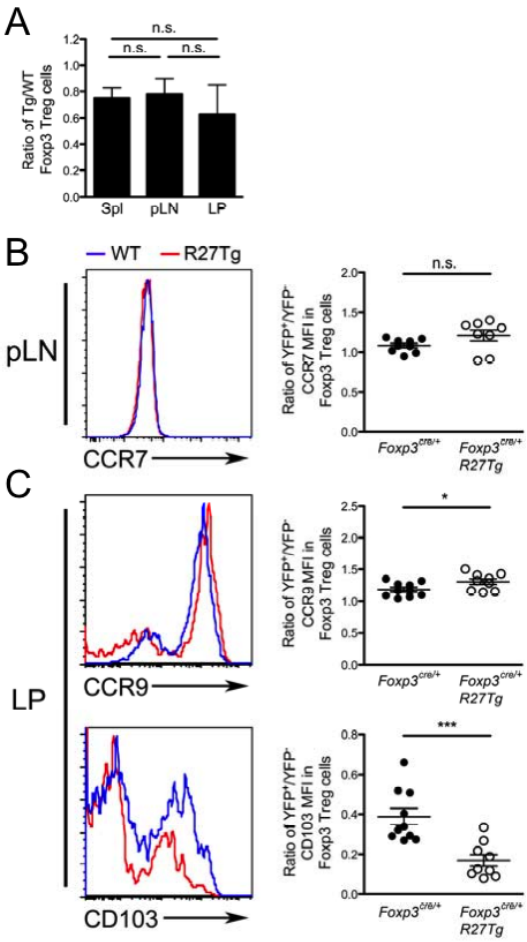

Figure 2.9: **Minimal role of miR-27 in controlling Treg cell tissue trafficking.** A) Ratios of YFP^{cre+} miR-27-overexpressing Treg cells and YFP^{cre-} WT Treg cells in indicated tissues from Foxp3$^{cre/+}$ R27Tg mice. FACS analysis and ratios of MFI of (B) CCR7 in pLN Treg cells or (C) CCR9 and CD103 in LP Treg cells with or without miR-27 overexpression from Foxp3$^{cre/+}$ R27Tg mice and WT control mice. Data represent mean ± SD and are representative of three independent experiments. Each symbol represents an individual mouse, and the bar represents the mean. *p <0.05, ***p <0.001

miR-27 targets c-Rel, a member of NFκB transcription factors crucial for Treg cell differentiation and homeostasis

While most research efforts on miR-27 to date have been primarily focused on its role in tumorigenesis or embryonic stem cell differentiation [39, 81, 104, 123], many miR-27 targets identified in those studies such as Foxo1 [52, 98], Runx1 [99, 109] and Smad2/3, were known to regulate Treg cell biology, too. We found that, while Foxo1 did not seem to be repressed by miR-27 in T cells, markedly diminished Smad2/3 and Runx1 protein levels could be detected in T cells that overexpressed miR-27 (Figure 2.10). Impaired TGFβ signaling resulting from miR-27-mediated repression of Smads likely contributed to the impaired iTreg cell differentiation phenotype described in our previous study [18]. On the other hand, despite a well established role of Runx1/CBFβ complex in maintaining Foxp3 expression [56, 109], excessive miR-27 expression only led to a marginal reduction in Foxp3 expression in the peripheral Treg cells (Figure 2.11), suggesting that the remaining Runx1 protein amount was sufficient to maintain Foxp3 expression in Treg cells.

Neither Smad2/3 nor Runx1 has been clearly shown to regulate thymic Treg cell development or peripheral Treg cell homeostasis [28, 122]. To gain further molecular insights into the mechanistic aspect of how miR-27 controls Treg cell differentiation and homeostasis, we first re-analyzed the recently reported RNA sequencing (RNA-seq) data of R27Tg T cells [18]. Gene ontology (GO) enrichment analysis demonstrated that excessive miR-27 expression resulted in significant changes in genes related to immune responses and hemocyte differentiation (Figure 2.12A). Additional screening of genes associated with Treg cell differentiation further revealed that Rel (the gene that encodes c-Rel), Nr4a1-3 and Foxo3 as well as the aforementioned miR-27 target genes Smad2 and Smad3 were downregulated in T cells with miR-27 overexpression (Figure 2.12B). c-Rel, Nr4a proteins and Foxo3 have all been shown to play crucial roles in regulating thymic Treg cell development [29, 45, 76, 98, 106, 113, 144]. Moreover, even though miR-27 generally does not seem to repress

Treg cell-related genes at the mRNA level as demonstrated by our cumulative distribution frequency (CDF) plot analysis (Figure 2.12C), further analysis of the previously established high-throughput sequencing of RNAs isolated by cross-linking immunoprecipitation (HITS-CLIP) results has identified c-Rel as a potential miR-27 target (Figure 2.12D) [75]. Supporting this notion, our luciferase reporter studies confirmed that miR-27 can directly repress c-Rel (Figure 2.12E) and R27Tg T cells expressed significantly reduced amounts of c-Rel protein (Figure 2.12F). Consistently, many previously identified c-Rel targets were also downregulated in T cells with miR-27 overexpression [10] (Figure 2.12G). These findings strongly suggested that excessive expression of miR-27 could negatively impact Foxp3 induction and Treg cell development in the thymus through targeting c-Rel [106]. Moreover, compromised c-Rel-dependent gene regulation could further contribute to the impaired peripheral Treg cell homeostasis observed in mice harboring miR-27-overexpressing Treg cells [45].

miR-27-overexpressing Treg cells fail to control effector T cell activation and function

In addition to reduced Treg cell numbers, we next sought to examine whether excessive miR-27 expression would also lead to impaired Treg cell function. Despite displaying comparable *in vitro* suppression capacity (Figure 2.13), unlike WT Treg cells, co-transferred R27Tg Treg cells failed to protect mice from weight loss and colonic pathology in the $CD4^{+}CD45RB^{hi}$ transfer model of colitis [88] (Figure 2.14A and B). Moreover, significantly higher IFNγ mRNA and protein levels were detected in colons harvested from mice co-transferred with miR-27-overexpressing Treg cells compared to mice receiving WT Treg cells (Figure 2.14C and D). By contrast, transfer of R27Tg Tconv cells alone did not result in weight loss and colitis (Figure 2.15 and data not shown). These results not only confirmed the aforementioned negative role of miR-27 in Teff cell homeostasis and function but also demonstrated that excessive miR-27 expression impaired Treg cell-mediated regulation of Teff

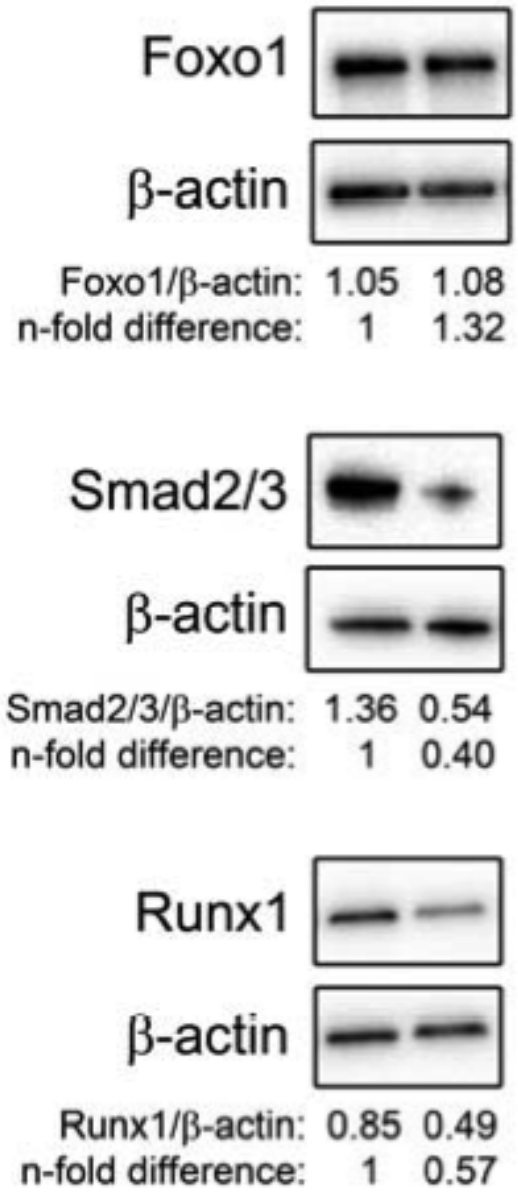

Figure 2.10: **Protein expressions of previously identified miR-27 targets in T cells.** Immunoblot analysis of Foxo1, Smad2/3 and Runx1 expression in T cells with or without miR-27 overexpression. Densitometric expression values of each molecule were normalized to β-actin expression values and n-fold increase on the basis of each corresponding WT. Data are representative of three independent experiments (n = 3-6).

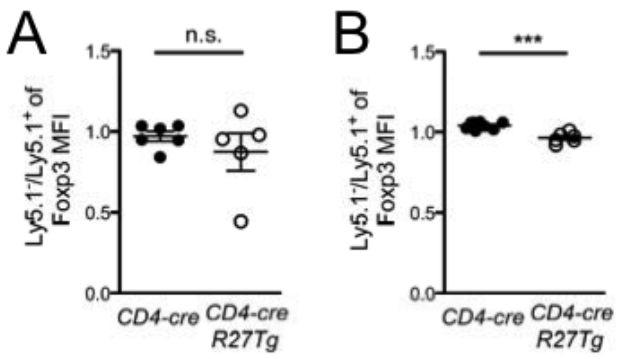

Figure 2.11: **Excessive miR-27 expression resulted in mild reduction in Foxp3 expression in peripheral Treg cells.** Ratios of MFI of Foxp3 between Ly5.1^{-} and Ly5.1^{+}(A) thymic or (B) splenic Foxp3^{+}CD4^{+} Treg cells. Data are representative of two independent experiments. Each symbol represents an individual mouse, and the bar represents the mean. ***p <0.001.

cell-driven intestinal autoimmunity. Nonetheless, it should be noted that mice receiving miR-27-overexpressing Treg cells also contained a significantly lower proportion of Foxp3^{+} cells at 8 weeks after co-transfer likely due to the aforementioned homeostatic defect. Therefore, it remained unclear as to whether the uncontrolled intestinal pathology in mice co-transferred with miR-27-overexpressing Treg cells was simply due to a lack of sufficient Treg cell numbers or the combination of reduced numbers and impaired suppressor function. To address this, we turned to hemizygous Foxp3cre R27Tg male mice. At young age, Foxp3cre R27Tg mice harbored similar Treg cell numbers compared to their WT littermates (Figure 2.14F), allowing us to directly examine the impact of miR-27 overexpression on Treg cell suppressor function in an *in vivo* setting. To this end, we could easily detect enhanced activation of splenic Teff cells with increased CD44^{hi}CD62L^{lo} cell subset and elevated production of IFNγ and IL-2 in Foxp3cre R27Tg mice compared to WT control littermates (Figure 2.14G and H). Similarly, while it is difficult to distinguish the difference in T cell activation in

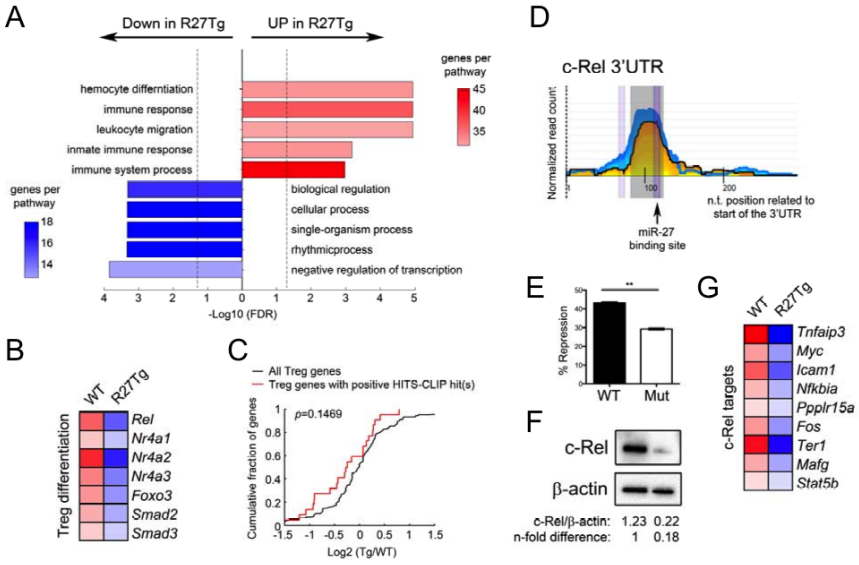

Figure 2.12: **miR-27 targets c-Rel and controls other genes important for Treg cell differentiation and homeostasis.**(A) Annotated gene ontology biological processes were assigned to genes differentially expressed in nave T cells with or without miR-27 overexpression as determined by RNA-seq. (B) Heat map of representative gene associated with Treg cell differentiation differentially expressed in the presence of excessive miR-27 expression. (C) Cumulative distribution frequency plots depicting the effect of overexpression of miR-27 on mRNA expression of Treg cell-associated genes. Levels of mRNAs of Treg cell-associated genes bearing HITS-CLIP identified miR-27 sites (red line) were compared with mRNAs of Treg cell-associated genes (black line). (D) HITS-CLIP analysis of putative miR-27 site in the 3'UTR of c-Rel. (E) Ratios of repressed luciferase activity of cells in the presence of c-Rel 3'UTR with or without mutations in the seed sequences in the presence of miR-27 compared with cells transfected with control miRNA were shown. (F) Immunoblot analysis of c-Rel expression in T cells with or without miR-27 overexpression. Densitometric c-Rel expression values were normalized to α-actin expression values and n-fold increase on the basis of each corresponding WT. (G) Heat map of representative c-Rel targeted genes differentially expressed in the presence of excessive miR-27 expression. Data represent mean ± SD and are representative of three independent experiments (n=3-6). **p <0.01.

LP between Foxp3cre R27Tg mice and their WT littermates as almost all LP T cells were already highly activated, elevated proliferation and increased production of inflammatory cytokine (i.e. IL-17) in T cells from LP could be easily detected despite having normal Treg cell numbers (Figure 2.16). These results strongly suggested that excessive expression of miR-27 is detrimental to Treg cell suppressor capacity to control Teff cell activation and function.

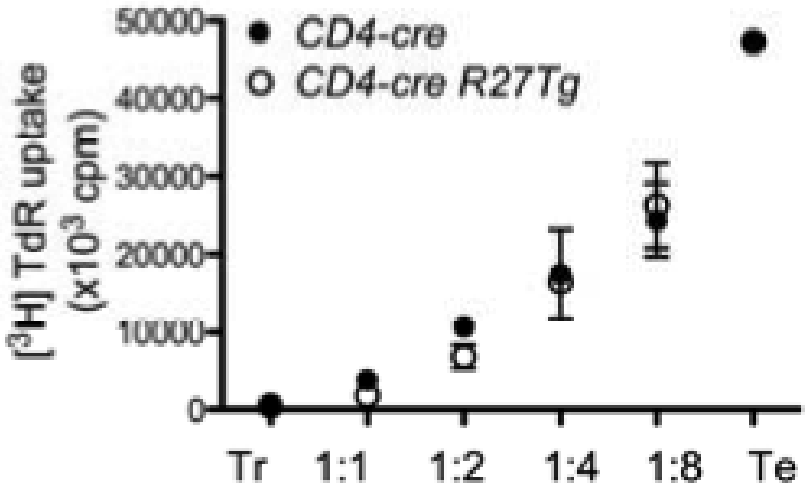

Figure 2.13: **Treg cells with miR-27 overexpression exhibited comparable *in vitro* suppressor capacity.**Treg cells (Tr) isolated from CD4- cre R27Tg mice or WT control littermates were subjected to *in vitro* suppression analysis at indicated ratios of responder T cells (Te). Data represent mean ± SD and are representative of three independent experiments (n=6).

Exaggerated miR-27-mediated regulation inhibits Treg cell function through repressing both known and novel targets

To explore the potential molecular mechanisms underlying miR-27-mediated regulation of Treg cell suppressor function, we first performed an unbiased transcriptom analysis of Treg cells that overexpressed miR-27 as well as other miR-23 family members. Similar to what was reported in Tconv cells [18], miR-27-overexpressing Treg cells again exhibited the most distinct global gene expression profile compared to other Tg Treg cell populations

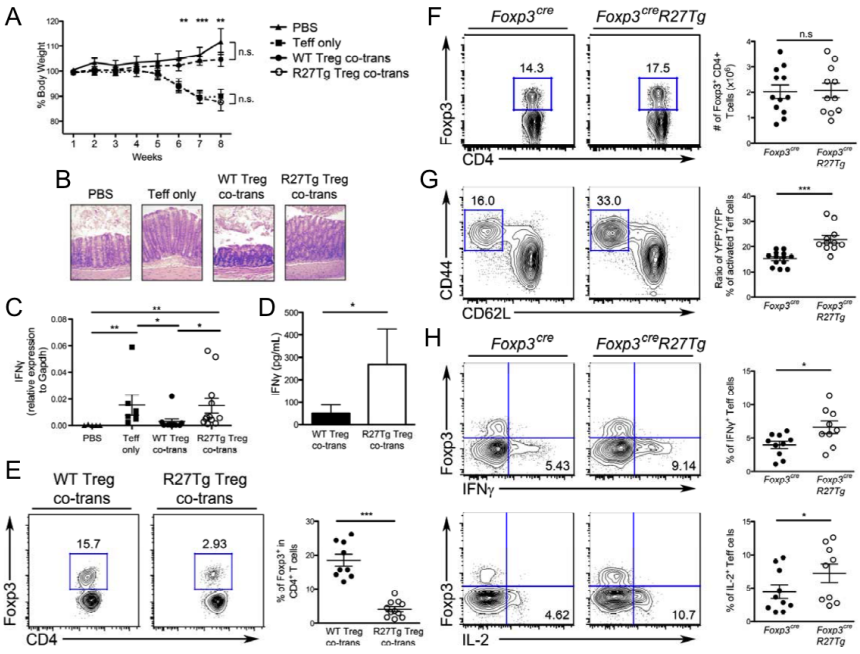

Figure 2.14: **Treg cells with miR-27 overexpression failed to maintain immunological tolerance.**(A) Percentages of body weight change of Rag1$^{-/-}$ recipient mice after adoptive transfer of 4 x 10^5 WT ($CD4^+CD45RB^{hi}CD25^-$) T cells with or without 2 x 10^5 WT or R27Tg Treg ($CD4^+CD25^{hi}$) cells. PBS injection only was used as no T cell transfer control. (B) 8 weeks after adoptive transfer, microcopy of colon sections from the mice stained with H and E. (C) qPCR and (D) ELISA analysis of IFNγ mRNA and protein levels in the colonic tissues harvested from indicated mice. (E) FACS analysis and frequencies of $Foxp3^+$ cells in total $CD4^+$ T cells isolated from lamina propria (LP). FACS analysis and frequencies of (F) $Foxp3^+$ cells in total $CD4^+$ T cells as well as (G) $CD44^{hi}CD62L^{lo}$ cells, (H) $IFN\gamma^+$ and $IL\text{-}2^+$ cells in Tconv cells from spleens in 6 wks old Foxp3cre R27Tg mice or WT controls. Data represent mean ± SD and are representative of three independent experiments. Each symbol represents an individual mouse, and the bar represents the mean. *p <0.05, **p <0.01, ***p <0.001.

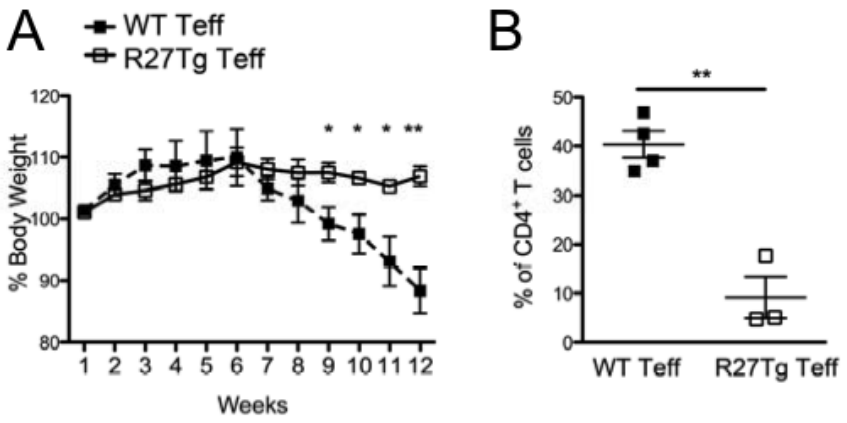

Figure 2.15: **Transfer of miR-27-overexpressing Tconv cells failed to induce colitis.** (A) Percentages of body weight change of $Rag1^{-/-}$ recipient mice after adoptive transfer of 4 x 10^5 ($CD4^+CD45RB^{hi}CD25^-$) WT or R27Tg T cells. (B) Frequencies of $CD4^+$ T cells isolated from lamina propria (LP) 12 wks after T cell transfer. Data represent mean ± SD and are representative of three independent experiments. Each symbol represents an individual mouse, and the bar represents the mean. **p <0.01.

(Figure 2.17A). Moreover, while it is not unexpected that miR-27 could mediate its effects in a cell-type specific manner similar to what was shown in other miRNAs [78], there was actually a strong positive correlation (r = 0.6383) in genes that were differentially expressed in Treg cells vs. Tconv cells in the presence or absence of miR-27 overexpression (Figure 2.17B), suggesting miR-27 could target many gene pathways shared by Treg and Tconv cells. The fact that excessive miR-27 expression negatively impacted homeostasis of both Treg and Tconv cells, as demonstrated by our mixed BM chimeras study, further supported this notion. Nevertheless, to directly assess the cell-intrinsic impact of excessive miR-27 expression on Treg cells without the potential environmental influences in CD4-cre R27Tg mice that would develop autoimmunity over time, we performed another transcriptome analysis of miR-27-overexpressing Treg cells isolated from the aforementioned heterozygous $Foxp3^{cre/+}$ R27Tg female mice. To this end, GO enrichment analysis identified genes related to immune system process were mostly affected by excessive miR-27 expression in Treg cells (Figure 2.18). Among them, many genes that are critical for Treg cell suppressor function were shown to be downregulated in Treg cells upon miR-27 overexpression (Figure 2.17C),

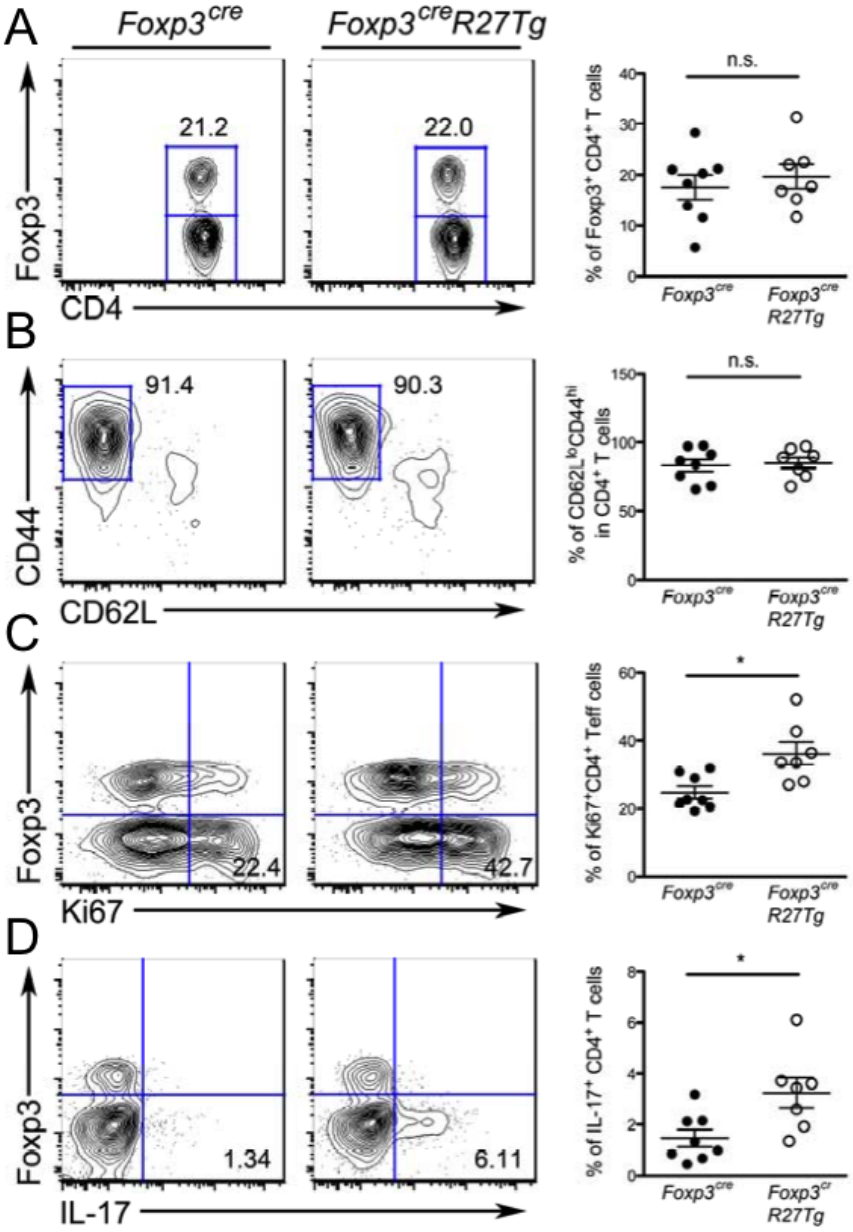

Figure 2.16: **Foxp3cre R27Tg mice exhibited elevated gut inflammation despite having normal Treg cell numbers.** FACS analysis and frequencies of (A) Foxp3$^+$ cells in total CD4$^+$ T cells as well as (B) CD44^{hi}CD62L^{lo} cells, (C) Ki67$^+$ and (D) IL-17$^+$ cells in Tconv cells from LP in 6 wks old Foxp3cre R27Tg mice or WT controls. Data represent mean ± SD and are representative of three independent experiments. Each symbol represents an individual mouse, and the bar represents the mean. *p <0.05.

providing the molecular basis for the autoimmune phenotypes observed in mice harboring miR-27-overexpressing Treg cells.

Next, by cross-analyzing our RNA-seq data and previous HITS-CLIP results, we sought to identify miR-27 target(s) that could contribute to the impaired suppressor function detected in Treg cells with exaggerated miR-27-mediated regulation. Although miR-27 in Treg cells on the whole does not seem to regulate its targets at the mRNA level (Figure 2.17D), HITS-CLIP analysis nevertheless revealed that IL-10 and GZMB, two well-established Treg cell suppressor molecules [12, 33, 107], could be directly inhibited by miR-27 (Figure 2.17E and F). Previously, it has already been reported that miR-27 can regulate macrophage-mediated inflammatory responses through targeting IL-10 [138]. Our luciferase reporter results has further demonstrated that like IL-10, GZMB can also be directly repressed by miR-27 (Figure 2.17G). Consistent with these findings, reduced frequencies of IL-10- and GZMB-expressing Treg cells with diminished IL-10 and GZMB protein production on a per-cell basis could be easily detected in the miR-27-overexpressing Treg cells compared to the WT counterparts in the same Foxp3$^{cre/+}$ R27Tg mice or in their WT littermates (Figure 2.17H and I). Similar results could also be obtained when TGFβ-dependent iTreg cells were examined (Figure 2.19). Together, our study identified known (i.e. IL-10) as well as previously uncharacterized (i.e. GZMB) miR-27 targets that could play critical roles in miR-27-mediated regulation of Treg cell suppressor function.

2.4 Discussion

Aberrant expression of miRNAs has been linked to a broad range of human diseases including both solid and liquid cancers, as well as metabolic, neurological and various immunological disorders [22, 50, 72, 102, 115, 130]. However, while distinct miRNA expression patterns serve as valuable biomarkers for disease diagnosis and prognosis, miRNA-based

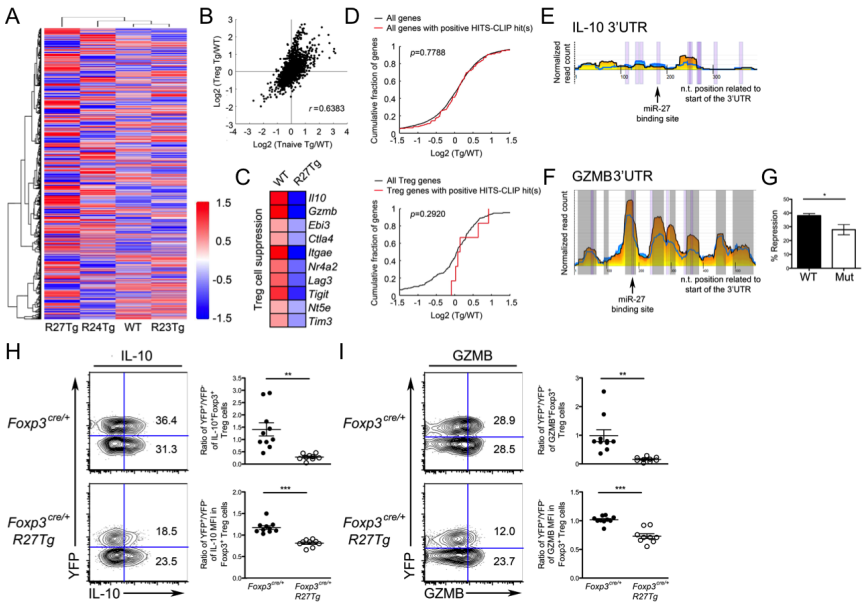

Figure 2.17: **Multiple Treg cell suppressor molecules including IL-10 and GZMB are regulated by miR-27.** (A) Clustering of RNA-seq results from R23Tg, R24Tg,R27Tg, and WT $CD4^{+}CD25^{hi}$ Treg cells based on total gene expression. (B) Scatter plot depicting log2 fold changes of gene expression in nave R27Tg Tconv cells over WT Tconv cells versus R27Tg Treg cells over WT Treg cells. (C) Heat map of representative gene associated with Treg cell suppressor function differentially expressed in YFP^{cre+} Treg cells isolated from either $Foxp3^{cre/+}$ R27Tg or $Foxp3^{cre/+}$ control mice. (D) CDF plots depicting the effect of overexpression of miR-27 on mRNA expression of Treg cell-associated or all genes. Levels of mRNAs of Treg cell-associated or all genes bearing HITS-CLIP identified miR-27 sites (red line) were compared with mRNAs of total Treg cell- associated or all genes (black line). HITS-CLIP analysis of putative miR-27 site in the 3'UTR of (E) IL-10 and (F) GZMB. (G) Ratios of repressed luciferase activity of cells in the presence of GZMB 3'UTR with or without mutations in the seed sequences in the presence of miR-27 compared with cells transfected with control miRNA were shown. FACS analysis and ratios of frequencies, and MFI of (H) IL-10 and (I) GZMB in LP $Foxp3^{+}$ Treg cells with (YFP^{cre+}) or without (YFP^{cre-}) miR-27 overexpression from $Foxp3^{cre/+}$ R27Tg and $Foxp3^{cre/+}$ control mice. Data represent mean ± SD and are representative of three independent experiments. Each symbol represents an individual mouse, and the bar represents the mean. *p <0.05, **p <0.01, ***p <0.001.

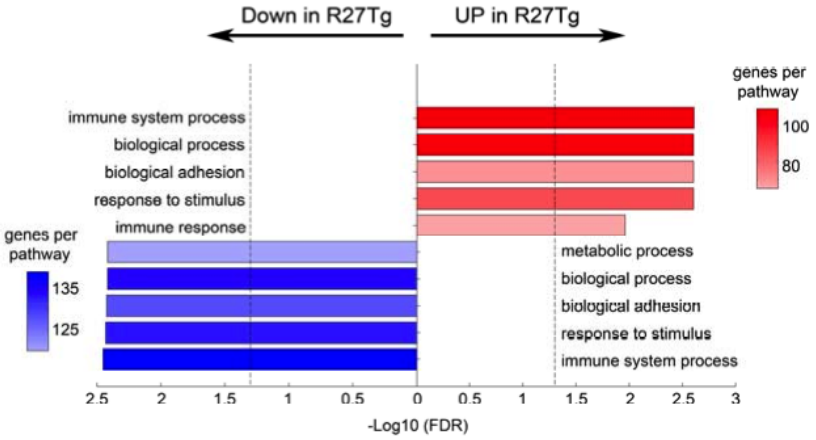

Figure 2.18: **Excessive miR-27 expression broadly impacted genes associated to immune system process in Treg cells.** Annotated gene ontology biological processes were assigned to genes differentially expressed in Treg cells with or without miR-27 overexpression as determined by RNA-seq.

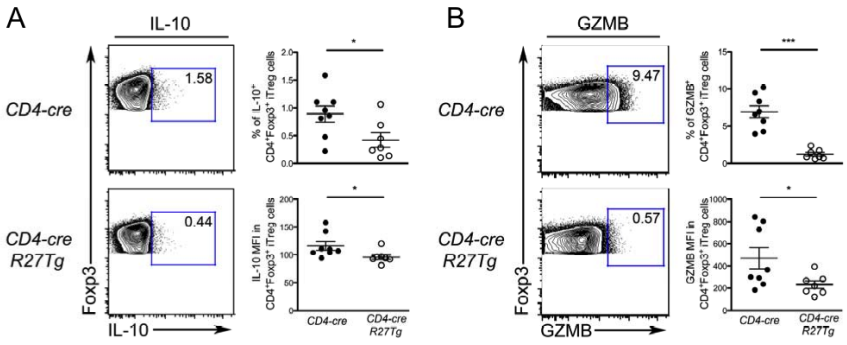

Figure 2.19: **Excessive miR-27 expression inhibited IL-10 and GZMB expression in iTreg cells.** FACS analysis of frequencies, and MFI of (A) IL-10 and (B) GZMB in R27Tg TGFβ-induced iTreg cells compared to WT controls. Data represent mean ± SD and are representative of three independent experiments. Each symbol represents an individual mouse, and the bar represents the mean. *p<0.05, ***p <0.001.

therapy is still in its infancy. In addition to overcoming the major challenge of effective delivery of miRNA or anti-miRNA constructs as potential therapeutic agents, understanding the precise role of a particular miRNA under a given disease setting is equally important to ensure a successful therapeutic outcome. To this end, it was recently reported that T cells from human MS patients exhibited high levels of miR-27 expression and that exaggerated miR-27-mediated gene regulation promoted proinflammatory IFNγ responses through repressing Bmi1-dependent Th2 differentiation [36]. In this study, by taking advantage of a mouse model in which T cells express elevated levels of miR-27 similar to those identified in human patients, we have provided direct experimental evidence to reconcile the seeming discrepancy in early studies regarding the impact of miR-27 on IFNγ-mediated Th1 responses and further identified a previously unappreciated role of miR-27 in regulating multi-faceted Treg cell biology.

Although miRNAs can certainly exert their biological function though the regulation of a single target [78], the effects of a given miRNA are usually subtle, and it is generally believed that miRNAs increase their impact through targeting a set of genes that are in a shared biological pathway [27]. To this end, many known miR-27 targets such as Foxo1, Runx1, Smad2/3 and IL-10 have already been shown to play key roles in controlling different aspects of Treg cell biology. Even Gata3, a miR-27 target that contributes to miR-27-dependent regulation of Th2 immunity [18], is also known for its role in maintaining Treg cell suppression function and identify [108, 133, 134]. In the current study, we have further demonstrated that miR-27 can directly repress c-Rel and GZMB, two molecules that are important for Treg cell development, homeostasis and suppressor function, respectively. While it is conceivable that there are more Treg cell-associated genes can be regulated by miR-27 either directly or indirectly, these findings unequivocally placed miR-27 as one of the key molecular regulators in controlling Treg cell biology.

Considering the detrimental effects of excessive miR-27 expression on Treg cell

homeostasis and function, it seemed counterintuitive that an miRNA normally expressed at higher levels in Treg cells compared to Tconv cell counterpart would play a negative role in controlling many aspects of their biology [18]. However, this is actually not the first molecule prevalently expressed in Treg cells that has been shown to be detrimental to the very same cell population when it is overexpressed. For example, while elevated T-bet expression is required for Treg cells to limit Th1 inflammation [57], under extreme Th1-polarized conditions during *T. gondii* infection or in the absence of cell-intrinsic negative regulators of the Th1 cytokine signaling pathway, Treg cells that acquired "supraoptimal" T-bet expression were shown to promote rather than suppress Th1 responses [77, 95]. Moreover, this phenomenon is not only restricted to transcription factors in Treg cells but was also reported with miRNAs in other immune cell types. To this end, while miR-146a is generally considered to be an anti-inflammatory miRNA as mice with miR-146a ablation developed spontaneous autoimmune disorders [7], forced expression of miR-146a in mice also resulted in autoimmunity [37]. It was suggested that different genes regulated by miR-146a were responsible for the seemingly contradictory phenotypes between Tg and KO mice. Thus, it is not surprising that miR-27 would also need to be properly controlled and that expression of miR-27 at either sub- or supra-optimal levels in Treg cells could perturb their function. Indeed, while we were not able to delete only miR-27 in Treg cells due to its genomic proximity to the other members in the miR-23˜27˜24 family, Treg cells devoid of this miRNA family also exhibited impaired suppressor function. Consequently, mice with Treg cell-specific ablation of the miR-23˜27˜24 family developed dysregulated IFNγ responses similar to what was observed in mice harboring Treg cells with excessive miR-27 expression, further supporting the aforementioned scenario (C.J. Wu and L.F Lu, unpublished results).

Our findings of impaired thymic Treg cell development in mice with T cell-specific miR-27 overexpression suggests that optimal miR-27 expression not only is required to safeguard Treg cell function in the periphery, but also pivotal to maintain normal Treg

cell differentiation in the thymus. The fact that a marked drop in miR-27 levels in Foxp3$^-$ CD4SP cells compared to DP thymocytes further supports the notion that miR-27 expressed at diminished levels immediately before Treg cell differentiation might be critical for their development in the thymus. Interestingly, among different members of the miR-23˜27˜24 family, only miR-27 plays a negative role in regulating thymic Treg cell development. While miR-23 does not appear to be involved in this biological process, miR-24 might even promote Treg cell differentiation. Coincidently, unlike miR-27, no significant change in the expression of miR-24 could be detected between DP and Foxp3$^-$ CD4SP thymocytes. Therefore, while the entire miR-23˜27˜24 family is upregulated in Foxp3$^+$ thymic Treg cells similar to what was observed in the peripheral Treg cells as described previously [18], our results suggested that the expression of each miR-23˜27˜24 family member can be differentially regulated prior to Foxp3 induction to ensure proper Treg cell differentiation in the thymus.

Previously, it has been shown that miR-27 could attenuate T cell responses through targeting IFNγ directly [13, 38]. Therefore, it was initially puzzling as to why dysregulated IFNγ responses were observed in both human MS patients and mice harboring T cells with excessive miR-27 expression [18, 36]. In addition to its negative impacts on TGFβ-mediated iTreg differentiation as previously shown by us and others [18,114], here we demonstrated that without directly impacting Foxp3 expression, exaggerated miR-27-mediated gene regulation severely impaired Treg cell-mediated immunological tolerance and promoted T cell-mediated autoimmunity even when Teff cells themselves were impeded by miR-27 overexpression. Moreover, our experiments have offered mechanistic insights into miR-27-mediated regulation of muti-faceted Treg cell biology. The findings obtained from this study will undoubtedly facilitate the development of proper therapeutic strategies for treating human immunological diseases.

2.5 Materials and Methods

Mice and experimental design

CD4-cre R23Tg, CD4-cre R24Tg, CD4-cre R27Tg, and CD4-cre R23cTg mice [18] as well as Foxp3Thy1.1 reporter mice [74] were described previously. R27Tg mice were breed to Foxp3cre mice [107] to obtain mice with Treg cell-specific overexpression of miR-27. Unless otherwise indicated, 6-12 wks old mice of both genders were used. Nevertheless, only WT littermates of the same gender served as controls in each individual experiment. Finally, all mice were on C57BL/6 background and maintained as well as 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

Single-cell suspensions of thymus, spleen as well as lymphocyte isolation in the lamina propria were prepared as described previously [18]. Cells were stained with Ghost Dye Red 780 (cat:13-0865-T100, Tonbo Biosciences) followed by surface and intracellular antibody staining including, CD4 (cat: 45-0042), CD8 (cat: 48-0081), CD44 (cat: 25-0441), CD62L (cat: 12-0621), CD103 (cat: 48-1031), CCR7 (cat: 12-1971), CCR9 (cat: 25-1991), Foxp3 (cat: 53-5773), and Ki67 (cat: 51-5698) at the recommended concentrations according to manufacturer's protocol (all from eBiosciences). Fixation and permeabilization of cells were performed with reagents from the Tonbo Biosciences Foxp3/transcription factor staining kit (cat:TNB-0607). To detect IFNγ (cat: 17-7311), IL-17 (cat: 48-7177) and IL-2 (cat: 25-7021) cytokine production (all from eBiosciences), 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. To detect GZMB (cat: 50-8898) and IL-10 (cat: 12-7101) production, total splenocytes were stimulated with plate-bound

αCD3 (cat:B E0001) and αCD28 (cat: BE0015) both at 1 ug/mL (from Bio-X-Cell) plus TGFβ (5ng/mL) and IL-2 (100U/mL) for 72 hours at 37C. Upon harvesting, cells were re-stimulated for 4 hours with PMA, ionomycin, and Brefeldin A as described above before staining. To measure *in vitro* cell survival, total splenocytes from $Foxp3^{cre/+}$ R27Tg mice or $Foxp3^{cre/+}$ control females were stimulated with plate-bound αCD3 and αCD28 (both at 1 ug/mL) for 6 hours at 37C followed by staining of Ghost Dye Red 710 (Tonbo Biosciences) as well as antibodies against CD4, Foxp3 and YFP/GFP (cat: 50-6498, eBiosciences). BD LSRFortessa or BD LSRFortessa 20x cell analyzer (BD Biosciences) was used for data collection while Flowjo software (Tree Star) was used for data analysis.

Generation of Mixed Bone-Marrow Chimeras

T cell-depleted BM cells isolated from femurs and tibias of $Ly5.1^{-}$ $CD4^{cre}$ R27Tg mice or WT littermates were mixed at 1:1 ratio with T cell-depleted BM cells taken from $Ly5.1^{+}$ WT mice (2.5×10^6 each) and i.v. injected into irradiated (950 cGy) $Rag1^{-/-}$ mice. Mice were kept on antibiotic water for 4 weeks and harvested for experiments 8 weeks after injection.

In vitro suppression assay

FACS purified 4×10^4 naive $CD4^{+}CD25^{-}CD62L^{hi}$T cells and $CD4^{+}CD25^{hi}$ Treg cells isolated from $CD4^{cre}$ R27Tg mice or WT control littermates were mixed at the indicated ratios and stimulated with 1ug/ml α-CD3 antibody in the presence of irradiated (2,000 rads) splenocytes. T cell proliferation was assessed by with 3H-TdR incorporation (cpm) in triplicate cultures during the last 8 hours of culture.

Adoptive T cell transfer colitis

To induce colitis, 4 x 10^5 $CD4^+$ $CD25^-$ $CD45RB^{hi}$ T cells from either $CD4^{cre}$ R27Tg mice or WT littermates were injected into $Rag1^{-/-}$ recipients i.p. as described previously [88]. To examine Treg cell-mediated protection from colitis, 4 x 10^5 $CD4^+$ $CD25^-$ CD45 RB^{hi} T cells from WT mice were mixed with 2 10^5 $CD4^+CD25^{hi}$ Treg cells from $CD4^{cre}$ R27Tg mice or WT littermates and i.p. injected into $Rag1^{-/-}$ recipients. Mice were sacrificed at indicated time points or before reaching below 80% of the original body weight. The colon was flushed with PBS and the concentration of IFNγ in the colonic supernatant was measured with an ELISA kit (cat: 43081, BioLegend) according to the manufacturer's instructions. Colonic tissue was saved for histology as well for RNA isolation and qPCR. Colonic cells were isolated for flow cytometry analysis as described above.

Histology

To assess immunopathology, different tissues were harvested and immediately fixed in 10% formalin solution. Paraffin-embedded sections were cut (5 mm) and stained with haematoxylin and eosin as described previously.

Quantitative PCR analysis

For quantification of the expression of miR-23˜27˜24 in different thymocyte subsets, DN, DP, CD8SP, $Foxp3^{Thy1.1-}$CD4SP and $Foxp3^{Thy1.1+}$CD4SP from $Foxp3^{Thy1.1}$ reporter mice were sorted on BD FACSAria II (BD Biosciences) with a purity of >95%, followed for RNA isolation using miRNeasy kit (Qiagen). TaqMan MicroRNA Assay (Thermo Fisher Scientific) was then performed as described previously [18]. For detecting IFNγ levels in the colon, colonic tissues were harvested and RNA was isolated using samples Trizol RNA kit (Thermo Fisher Scientific). Extracted RNA was converted to cDNA with iScript cDNA Synthesis Kit (Bio-Rad) followed by qPCR reactions using SYBR select Master Mix (Thermo

Fisher Scientific). All real-time reactions were run on 7900HT Fast Real-Time PCR System.

Immunoblotting

FACS purified naive $CD4^+$ T cells were subjected to lysis with RIPA buffer supplemented with 1 mM PMSF for 20 minutes. Cell debris was removed by centrifugation at 14,000 rpm for 15min at 4C, and the supernatant were transferred to fresh tubes. Cell lysates were separated by SDS-PAGE followed by a PVDF membrane (Bio-Rad Laboratories) transfer. Antibodies against β-actin (cat: AC-74, Sigma-Aldrich), SMAD2/3 (cat: ab3862, Chemicon), Foxo1 (cat:2880, Cell Signaling), c-Rel (cat: 14-6111, eBioscience), and Runx1 (cat: 255019, R&D Systems) were used to visualize the corresponding proteins. Quantification of proteins was calculated with ImageJ (National Institutes of Health).

Luciferase Reporter Assay

The 3'UTR region of Rel or Gzmb were cloned into psiCheck2 (Promega). Plasmids containing mutated miR-27 binding sites located on the 3'UTR of Rel or Gzmb, were generated by site-directed mutagenesis (Agilent). WT or mutated psiCHECK2 constructs were transfected along with control miRNA (miR-155) or miR-27 expressing pMDH-PGK-EGFP plasmid using FuGENE 6 Transfection Reagent (cat: E2691, Promega) into 293T cells (ATCC). 24 hours post transfection, luciferase activity was detected with Dual-Luciferase Reporter assay system (cat: E1960, Promega) as described previously [18].

Gene expression profiling analysis

$CD4^+CD25^+$ or $CD4^+Foxp3^-YFP^+$ Treg cells were FACS sorted from 6 week old indicated $CD4^{cre}$ driven Tg mouse lines or heterozygous $Foxp3^{cre/+}$ R27Tg female mice, respectively. Poly-A RNA-sequencing was performed using three biological replicates for each cell population similar to what was described previously [18]. Genes up-regulated

or down-regulated were identified with a 1.5 log2 fold change in transgenic naive T cells (GSE75909) or Treg cells relative to WT using the median expression across 3 replicates in each condition. Gene expression clustering was performed using Cluster 3.0 and visualized using Java TreeView. GO biological processes associated with each set of up/down regulated genes were queried, and the top 5 GO categories with the most significance were selected. Significance of the GO gene enrichment was calculated by first using a fishers exact test to test enrichment, and then corrected p-values using Benjamini-Hochberg FDR [6]. Scatter plot analysis was performed to measured the differences between Tconv and Treg cells in the presence or absence of miR-27 overexpression. The Pearson correlation coefficient was calculated to determine how the the log2 ratios of (Tconv Tg/WT) and (Treg Tg/WT) are related with each other. CDF plot analysis was performed as described previously [18]. Briefly, target sites were restricted to perfect seed complementarity between positions 2 and 7 of the corresponding miRNA with positive Argonaute binding peaks in the HITS-CLIP database [75]. Empirical cumulative distributions were computed using Matlab (R2014b) to display the log2(miRNA Tg/WT) against the cumulative frequency of all genes or Treg cell-associated [119]. RNA-seq data for Treg cells from CD4-cre driven Tg lines and heterozygous Foxp3$^{cre/+}$ R27Tg females are available from NCBI under accession no. GSE89548.

Statistical Analysis

Unpaired Student's t tests (or one way ANOVA tests for IFNγ expression in adoptive T cell transfer colitis studies) were performed using Prism software (GraphPad). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ in all data.

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Author contributions

L.O.C. and L.-F.L. conceived and designed the project. L.O.C., C.-J.W., S.C. and L.-L.L. performed the experiments and acquired data. L.O.C., S.S.H., A.A.K. and L.-F.L. analyzed the data. L.O.C., D.T.N. and L.-F.L. wrote the manuscript.

Chapter 2, in full, is in press. "Excessive miR-27 expression impairs regulatory T cell-mediated immunological tolerance", Leilani Ong Cruz, Somaye Sadat Hashemifar, Cheng-Jang Wu, Sunglim Cho, Duc T. Nguyen, Ling-Li Lin, Aly Azeem Khan and Li-Fan Lu. *The Journal of Clinical Investigation* (In press) The author of this dissertation was the first investigator and author of this paper.

PERSPECTIVES

miRNAs have been studied as potential biomarkers in diagnosis of human diseases. Recent advances have shown that they function as crucial regulators in multiple biological processes. Among them, miR-23a/b clusters have been studied quite extensively in many human cancer models and have recently been shown to have a role in driving autoimmune diseases [16]. Nevertheless, miR-23a/b clusters have not been well-studied in the context of T cell immunity. In this work, we first identified a unique differential expression pattern of the miR-23a/b clusters in Treg vs. Tconv cells. Through genetic, biochemical, and immunological approaches with whole animal experimentation, we have further demonstrated how miR-23a/b clusters and their individual members can control multiple aspects of T cell biology.

Given the fact that miR-23a/b clusters are expressed at low levels in Tconv cells, we hypothesized that tight regulation of this miRNA family is required for proper T cell development, Th cell differentiation and effector function. To investigate this, we utilized gain-of-function genetic models enabling the overexpression of the miR-23 cluster, as well as the individual members, specifically in CD4-expressing T cells. Forced expression of this miR-23 cluster in T cells resulted in dysregulated T cell activation and autoimmune inflammation. Moreover, in addition to a general impact on T cell activation, the miR-23a/b clusters also play a central role in T cell differentiation. In particular, different members of the miR-23 family cooperate to control Th2 immunity by targeting IL-4 and Gata3, respectively. Intriguingly, we also found that in modulating the differentiation of other Th lineages,

different members of this miRNA family actually antagonize rather than cooperate with each other. Furthermore, results obtained from loss-of-function studies with T cell-specific ablation of both miR-23a/b clusters supported our overexpression findings and provided clinical relevance as mice harboring T cells devoid of both miR-23a/b clusters exhibited enhanced Th2-driven eosinophilic airway inflammation upon OVA-challenge. Collectively, the study described in Chapter 1 represents the first in-depth description of the immunological function of this important miRNA family whose activities will likely be of significant therapeutic value in modulating T-cell mediated immunological diseases.

As mice with T cell-specific overexpresion of a single miR-23 family member, miR-27, phenocopied most of the phenotypes observed in mice harboring miR-23 cluster transgenic T cells, we hypothesized that miR-27 is the dominant driver of the miR-23 cluster and therefore decided to further pursue an in-depth characterization of miR-27 transgenic mice in Chapter 2. Our study revealed that excessive miR-27 expression in T cells led to the development of autoimmunity disorders. However, the observed dysregulated T cell response in these mice was not due to a cell-intrinsic role of miR-27 in promoting Teff cell activation, and cytokine production but rather resulted from an impaired Treg cell compartment. Mechanistically, by using a combination of RNA-seq and HITS-CLIP analysis we show that many genes associated with Treg cell development and suppressor function are directly repressed by miR-27. Specifically, we identified two novel miR-27 targets; c-Rel, which is responsible for proper Treg cell development and homeostasis, and GZMB, a known Treg cell suppressor molecule. In addition to c-Rel, our genome wide transcriptome study combined with HITS-CLIP analysis further revealed that atypical inhibitor of NFκB called IκBNS (inhibitor of κB) encoded by NFκBID gene, could also be a direct miR-27 target. Like studies in T cells with c-Rel deficiency, mice with T cells devoid of IκBNS also exhibited reduced Treg cell numbers [112]. While further investigation is required to formally test this possibility, these studies strongly suggested that the observed impaired Treg cell development could be due

to a combinatory effect from exaggerated miR-27-mediated repression of both c-Rel and IκBNS.

Our findings clearly pointed to an important role of miR-27 in regulating different aspects of Treg cell biology, however it was initially puzzling as to why a miRNA that is normally expressed at higher levels in Treg cells compared to their Tconv cell counterpart would play a negative role in controlling many aspects of their biology [37, 77, 95]. However, this is actually not the first molecule prevalently expressed in Treg cells that has been shown to be detrimental to the very same cell population when it is overexpressed. It is thus not surprising that miRNA expression also needs to be tightly regulated to ensure proper regulation and at either sub- or supra-optimal levels could perturb their function. Supporting this notion, while it is very difficult to singly delete miR-27 in the miR-23 cluster due to the very close proximity of different members on the primary transcript, our preliminary findings (Wu et al. unpublished data) revealed Treg cells devoid of the entire miR-23 family are also functionally compromised. Collectively, in Chapter 2, we concluded that optimal miR-27 expression is essential to properly control Treg cell development, homeostasis and suppressor function by targeting a network of Treg cell-related genes. In addition to miR-155 [80] and miR-146a [77] which have been previously shown to play important roles in maintaining normal Treg cell homeostasis and their suppression of Th1 inflammation, respectively, we now identify miR-27 as another miRNA critical for controlling multifaceted Treg cell biology.

Despite the fact that a single miRNA can interact with hundreds of target genes forming a network of gene regulation, it has been thought that only a small number of key target genes mediate the function of each miRNA [2, 34]. Nevertheless, to gain further insights into miRNA-mediated immune regulation, it is absolutely critical to identify those central targets responsible for the observed phenotypes. Our RNA-seq-HITS-CLIP approaches have been proven to be useful allowing us to confirm several well-studied gene targets as well as identify novel targets of the miR-23 cluster in controlling T cell immunity.

However, because biochemical identification of Ago binding sites came from high-throughput sequencing analysis of pooled mRNA after Ago immunoprecipitation, there was no easy way to identify the corresponding miRNAs responsible for Ago binding and many potential important miRNA targets might be missed due to the lack of clear miRNA seed matches. To this end, alternative sequencing techniques such iCLIP, could provide additional resolution by sequencing the direct miRNA:mRNA interaction through an additional ligation step that connects the interacting miRNA and mRNA pair during the original HITS-CLIP procedure [44]. Thus, it will be useful to conduct the aforementioned iCLIP analysis in T cells as well as other immune cell types to gain clearer understanding of the role of miRNA in immune system.

Studies conducted by us and others have revealed crucial roles of miRNAs in immunological and other biological processes. Moreover, the fact that aberrant miRNA expression has been found to be highly associated with many human diseases has further led to considerable interest in developing new therapeutic strategies through miRNA targeting. Currently, there are three main approaches to manipulate miRNA expression or function. First, miRNA sponges: this vector-based strategy utilizes oligonucleotide mRNA constructs containing multiple artificial miRNA-binding sites in tandem located downstream of a reporter gene, which acts as decoys or 'sponges'. When introduced into the cell, sponges will 'soak up' endogenous miRNAs, decreasing miRNA expression levels. Second, small-molecule inhibitors: small-molecule inhibitors can target three different stages of miRNA assembly and function. They can interfere with transcription of the pri-miRNAs, pri-miRNAs processing, and interactions between RISC and target mRNA. And finally antisense oligonucleotides (anti-miRs): anti-miRs are designed to be complementary to the target miRNA. When delivered into cells, the anti-miRs bind to the target miRNA thus relieving repression of the endogenous target genes. While finding an effective delivery method of these therapeutic agents into a specific cell type remains a major challenge, currently many compounds based

on the aforementioned approaches are in now preclinical and clinical development for various diseases such HCV infection, cancer and cardiac diseases [66].

To date, even though there are no published records of the members of the miR-23 cluster in any miRNA-related drug development, miR-27 has been implicated in regulating some very important pharmacogenes [142] as well as to enhance anticancer drug responses by activating p53-dependent apoptosis and reducing CYP1B1-mediated drug detoxification [89]. Moreover, as previously mentioned, the fact that isolated T cells from multiple sclerosis patients expressed high levels of miR-27 further suggests that miR-27-mediated gene regulation plays an important role in this autoimmune disease. Our finding in miR-27 could serve as a molecular basis to develop new therapeutics for treating this particular disease and other autoimmunity with dysregulated miR-27 expression. Finally, as the miRNA field continues to evolve, a better and more thorough understanding of miRNA biogenesis and function will help to ensure a successful therapeutic outcome from miRNA-based therapy for not only treating immunological disorders but also other human diseases.

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