UNIVERSITY OF CALIFORNIA, SAN DIEGO

A Non-redundant Role for Thymic Epithelial MicroRNA-155 in the Generation of Tregs

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by

Lindsey Marie Warner

Committee in charge:

Li-Fan Lu, Chair
Elina Zuniga, Co-Chair
Wenxian Fu

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The Thesis of Lindsey Marie Warner is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-Chair

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Chair

University of California, San Diego

2017

DEDICATION

To my family, friends, and loved ones:

To my mom, for always encouraging me to further my education and do what I love.

To my dad, for supporting me and reminding me that there is always time for fun.

To my sister, whose ongoing lesson is the value of competition and individuality.

And to Jack, for being my rock to and telling me to eat my vegetables.

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ABSTRACT OF THE THESIS

A Non-redundant Role for Thymic Epithelial MicroRNA-155 in the Generation of Tregs

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miR-155 is a non-coding RNA that is highly upregulated in regulatory T-cells (Tregs), the adaptive immune cells specialized in suppressing autoreactive T cells in order to maintain tolerance and homeostasis. While several studies have confirmed the importance of miR-155 in modulating IL-2R sensitivity to maintain Treg homeostasis, alternative pathways through which miR-155 regulates Treg development remain to be elucidated. In addition to its role in T cells, through taking cell-type specific gene

targeting approaches, we demonstrate that miR-155 in Thymic Epithelial Cells (TECs) is equally important in promoting thymic Treg (tTreg) development. In particular, we show that $CD80^{hi}MHC\ II^{hi}$ medullary TECs (mTECs), a mature mTEC subset crucial for the generation of tTregs, express high levels of miR-155 and that deletion of TEC-specific miR-155 led to a selective reduction in this mTEC population. Further investigation into the role of thymic epithelial miR-155 pointed to a potential mechanism whereby miR-155 targeting of SMAD3 attenuates TGF-β signaling in mTECs. As the role of TGF-β signaling in controlling the generation of mTECs has been previously established, here we propose a miR-155-SMAD3 axis that regulates TGF-β signaling in the thymic medulla to determine mTEC maturity and, in consequence, the quantity of developing tTregs.

INTRODUCTION

Chapter 1: Thymic Regulatory T cell Development

Regulatory T cells (Tregs) are key players in immune homeostasis and are essential in preventing immune responses against self-antigen and innocuous foreign-antigen. Expression of the master transcription factor Foxp3 in Tregs is crucial to their development and is well appreciated for its role in the induction of their suppressive faculties [1]. Ablation of Foxp3 in mice results in the manifestation of scurfy, which is characterized by systematic inflammation, hyperproliferation of effector T cells, and fatal autoimmunity usually by 3 weeks of age [1], [2]. For this reason, the correct development and differentiation of Tregs is necessary for maintaining self-tolerance and thwarting autoimmune disorders.

Central tolerance begins with the generation of thymus-derived regulatory T cells (tTregs) in discrete thymic microenvironments, namely the cortex and the medulla. During thymic development, immature $CD4^+CD8^+$ double-positive thymocytes undergo positive selection as mediated by cortical thymic epithelial cells (cTECs) to test TCR recognition of MHC class I or II before differentiating into $CD8^+$ and $CD4^+$ single-positive thymocytes, respectively [3], [4], [5]. These single-positive thymocytes are then subjected to negative selection in the medulla: TCR affinity for self-antigen presented by either medullary thymic epithelial cells (mTECs) or thymic resident dendritic cells is assessed, laying down the foundation for central tolerance.

In the medulla, T cells with a low affinity for self-antigen complete negative selection and become mature naïve single positive thymocytes that can migrate to the periphery, while T cells containing TCRs that bind strongly to self-peptide MHC

complexes are eliminated via apoptosis. This “affinity model of thymocyte selection” suggests an overlap where $CD4^+$ single positive T cells having some intermediate TCR strength for self-peptide can be redirected into the Treg lineage [3], [5]. While selection of tTregs is in part dependent on TCR self-reactivity, it is still unclear how the threshold for autoreactivity is actually determined.

In addition to the intensity of TCR self-reactivity, tTreg development depends on a combination of cell intrinsic and extrinsic cues for proper differentiation and function. Upregulation of the interleukin-2 receptor alpha chain (IL-2Rα, or CD25) and the activation inducer molecule CD69 in T cells has long been appreciated to be a consequence of TCR activation; CD25 and CD69 are required for increasing sensitivity to IL-2 (an essential Treg growth factor) and inducing proliferation, respectively [6], [7]. In particular, CD25 expression is fundamental to the production and maintenance of tTregs. Without induction of CD25 for the necessary IL-2/IL2R interactions, the proportion of tTregs and their $CD4^+Foxp3^-CD25^+$ precursors are negatively impacted: Tregs are functionally active, but unable to control autoreactive T cells due to their low numbers [8]. These signaling cues are inherently important for proper tTreg development and efficacy.

A combination of cell extrinsic signals provided by the thymic microenvironment also plays a large role in supporting tTreg survival, differentiation, and function. The expression of TGF-β by cTECs and apoptotic thymocytes has been implicated in the development of tTregs early in life [9], [10], [11], [12]. In the thymic medulla, mTECs impact both the generation and repertoire of tTregs through their expression of co-stimulatory ligands and tissue-restricted antigens (TRAs). The generation of tTregs and

their $CD4^+Foxp3^-CD25^+$ precursors depends on co-stimulation of CD28 by its ligands CD80/CD86, as loss of CD28 or these ligands results in failure of both survival and de novo generation of tTreg [13], [14]. In support of this phenomenon, it has recently been shown that RelB dependent mTECs are necessary for the development of tTregs but not conventional $CD4^+$ single positive T cells; RelB deficient TECs demonstrate a drastic reduction in the proportion of $CD4^+Foxp3^-CD25^+$ tTreg precursors, a defect that carries over into the mature tTreg pool [15], [16]. This provokes the idea that mTECs are responsible for determining a unique thymic microenvironment to support Treg development.

The size and maturity of mTECs can also directly impact both the quantity and quality of tTregs. mTEC maturation is typically characterized by the expression of co-stimulatory molecules CD80/CD86, MHC class II (MHC II), and autoimmune regulator AIRE [17], [4], [18]. The induction of these molecules in $CD80^-AIRE^-$ mTEC progenitors depends on the expression of TNF superfamily ligands CD40L and RANKL on innate lymphoid cells and mature thymocytes, where activation of RANK signaling in mTECs by these ligands will lead to the upregulation of CD80, MHC II, AIRE and, consequently, the expression of TRAs essential for shaping the Treg repertoire [17], [19], [20], [21]. Dysregulation of any of these molecules results in an mTEC population that cannot adequately promote the expansion of self-tolerant Tregs. For example, TGF-β signaling has been shown to restrict mTEC growth and differentiation, as constitutive TGF-β signaling limits the size of $CD80^{hi}$MHC II^{hi} mTECs, which diminishes the tTreg pool [22]. Additionally, Spi-B mediated osteoprotogerin (OPG) negatively feedbacks on mTEC development, reducing CD80 and AIRE expression and resulting in a limited Treg

repertoire [23], [24]. While these findings demonstrate the importance of mTEC differentiation and size in establishing developmental niches for central tolerance, it remains unclear how gene regulation within mTECs is determining the thymic microenvironment and to what extent tTregs are affected.

Chapter 2: microRNAs are Key Players in Immune Homeostasis

microRNAs (miRNAs) are a class of endogenous small non-coding RNAs that play major regulatory roles in both animals and plants by post-transcriptionally targeting genes for suppression or degradation [25], [26]. These short 21-25 long nucleotide RNAs can be differentially expressed to negatively regulate genes at the transcript or protein level during cellular growth, differentiation, and nearly every other cellular process [25], [27]. Since the discovery of pioneer miRNA lin-4 in *C. elegans*, micro-RNAs have become a hot topic in molecular biology due to their diverse regulatory capabilities and promising potential as therapeutic agents in human disease [28], [27], [29]. While the complex molecular mechanisms by which miRNAs target and modulate a myriad of genes continues to be elucidated, recent studies have demonstrated that inappropriate miRNA-mediated gene repression is implicated within a variety of human pathologies, including cancer, metabolic disorders, and many immunopathologies [29], [30], [31]. Further understanding of how miRNA-target interactions operate in a cell-specific context to determine a cell's metabolism, differentiation, or homeostasis will help to clarify the relationship between miRNAs and human disease.

The molecular characteristics that define miRNA effector functions are essentially identical to the biological processes of RNA interference (RNAi), where small interfering RNAs (siRNAs) similarly participate in gene repression at the transcript level. However, miRNAs are unique from siRNAs in that they are endogenously encoded in the genome. The biogenesis of miRNA begins with the synthesis of a primary miRNA (pri-RNA) transcript that is processed by RNase-III enzyme Drosha into an approximately 70-nucleotide long stem loop precursor miRNA (pre-miRNA) in the nucleus before being

exported to the cytoplasm [27]. Next, the pre-miRNA hairpin is cleaved by Dicer (another RNase-III enzyme), which generates a 21-25 nucleotide long miRNA:miRNA duplex consisting of the mature miRNA imperfectly bound to its complementary strand [27], [26]. This mature miRNA can now be incorporated into protein complexes in the cytoplasm that mediate post-transcriptional repression.

The effector complex that facilitates the miRNA-target interaction is the RNA-induced silencing complex (RISC), an assembly of proteins including the direct miRNA binding protein Argonaute (AGO). The mature miRNA in the newly generated miRNA:miRNA pair is selected for integration into AGO based on its thermodynamic stability: the miRNA strand having the least stable 5' arm in the duplex is preferentially selected by AGO [27], [32]. AGO then plays a crucial role in target recognition, as binding of the mature miRNA to AGO forces a conformational accommodation that exposes the miRNA's conserved 2-8 nucleotide seed sequence necessary for target recognition [32], [26]. With the seed sequence poised for recognition, RISC can guide the miRNA to the 3' UTR on its target mRNA transcript; the miRNA binds imperfectly to its target sequence and triggers translational repression [27], [32], [33]. Interestingly, the majority of animal miRNAs exhibit this imperfect target binding that results in translational inhibition, while plant miRNAs and siRNAs more commonly promote direct cleavage due to their near perfect binding to the complementary target sequences [27]. Therefore, miRNA-mediated gene repression is typically characterized in two ways: imperfect binding to the complementary target sequence that results in translational repression and diminished protein expression, or miRNA that binds perfectly and encourages degradation at the mRNA transcript level. In metazoans, this act of non-

specific binding gives a single miRNA the potential to have hundreds of target genes [32]. This phenomenon suggests that a single miRNA can participate in regulating the development and homeostasis of many cell types that may radically differ in function or origin.

While miRNAs are well appreciated for their regulatory roles in developmental pathways, recent investigations have demonstrated a particularly crucial responsibility for them in hematopoiesis and the proper function and maintenance of the immune system. This is most clearly observed under the conditional deletion of Dicer in hematopoietic stem cells (HSCs): these cells lose their capacity to reconstitute the HSC pool, which negatively impacts the downstream generation of erythroblasts and adaptive and innate immune cells [34], [35], [36]. Ablation of Dicer in B cells results in their impaired differentiation, expansion, and a compromised antibody repertoire [37], [35], [34]. Expression of the miR-17~92 cluster during B cell development is essential for suppressing proapoptotic factor BIM; without the proper Dicer-mediated miRNA processing of pre-miR-17~92, BIM expression is uncontrolled and B cell survival is notably impaired [37]. Furthermore, loss of Dicer in T cells results in their diminished development as well as skewed differentiation of T helper subsets and cytokine production [38]. Even TCR signaling responses can be fine-tuned by miRNAs; activation of CD69 leads to the induction of miR-17 and miR-20a, which ironically act to suppress CD69 expression [39]. Surely, miRNAs play quite crucial roles in shaping the development of an adaptive immune system.

Innate immune cells similarly rely on miRNAs for proper survival and function. For example, toll-like receptor (TLR) signaling in macrophages is modulated by miR-155

and miR-146a: miR-155 induction by TLR4 amplifies inflammatory signals by inhibiting SHIP1, while miR-146a expression by NF-κB dampens the TLR4 response by negatively regulating TRAF6 and IRAK1 [40], [41]. Taken together, these data demonstrate a fundamental requirement for miRNA in the growth, maintenance, and efficacy of cells comprising both the innate and adaptive the immune system.

In particular, miRNA profiling of T cells has revealed an interesting phenomenon where different helper T cell subsets differentially express miRNAs to determine and maintain their effector functions. Coordinated repression of PTEN, TGFB receptor II, and CREBI by members of the miR-17~92 cluster helps to promote T helper 1 (Th1) cell responses [42]. Also, recent studies have shown a role for miR-24 and miR-27 in limiting the activity of T helper 2 (Th2) cells by inhibiting Th2 differentiating cytokine IL-4 [43]. Even the pro-inflammatory functions of T helper 17 (Th17) cells that often potentiate autoimmune pathologies have been attributed to the expression or dysregulation of specific miRNAs; both miR-21 and miR-301a promote Th17 differentiation, and are highly expressed in mice during experimental autoimmune encephalomyelitis (EAE) [44], [45]. Clearly, these findings demonstrate that differential expression of miRNA helps to characterize T helper cell function and make major contributions to T cell development. This phenomenon of differential miRNA expression across immune cell populations has provided an exciting avenue of research that, while in its infancy, offers another platform for studying gene regulation that encourages new approaches for efficiently studying miRNA-mediated repression in cell specific contexts.

Chapter 3: microRNA-155 in Regulatory T cell Biology

Similar to how miRNA expression varies amongst T helper cells to promote their effector function, Tregs possess a miRNA profile drastically distinct from conventional $CD4^+$ T cells. Deletion of Dicer in developing double positive thymocytes results in a loss of Dicer-mediated miRNA processing and, interestingly, a striking reduction (greater than 50%) in the proportion of $Foxp3^+$ tTregs [46]. This implicated a role for miRNAs in regulating genes induced by Foxp3 that are required for Treg function, such as miR-146 [46]. In favor of this, miRNAs that are upregulated in activated conventional T cells are distinctly expressed in Tregs, suggesting that specific miRNAs can influence the expression of molecules like CD25, CD69, or CTLA4 that are transiently expressed in activated $CD4^+$ T cells but constitutively expressed in Treg [47], [46]. While the growing consensus is that the Treg miRNA profile mimics that of activated conventional T cells, precise studies to elucidate the targets of miRNAs that determine Treg differentiation are required to understand tTreg induction and maintenance in the periphery over time.

The unique miRNA profile Tregs have is in part conferred upon by transcription factor Foxp3, where Foxp3 acts as a transcriptional activator or repressor for hundreds of genes (including non-coding RNAs) involved in determining Treg fate. One miRNA of particular interest is microRNA-155 (miR-155), a miRNA that has been well implicated in hematopoiesis but is also highly expressed in Tregs due to Foxp3-dependent transcriptional activation [48], [34], [49]. miR-155 is processed from primary transcript BIC, which has long been associated with the development of viral-induced lymphomas. While miR-155 is typically induced in lymphocytes and other leukocytes following stimulation of antigen receptors or exposure to pro-inflammatory cytokines,

dysregulation of miR-155 is also prevalent in many immunopathologies. B cell lymphoma is notoriously associated with high levels of miR-155 [31], [50]. A broader role for miR-155 in other immune cells has also been demonstrated: miR-155 deficiency in dendritic cells (DCs) results in diminished T cell activation, and miR-155 deficient T cells cannot adequately respond to IL-2 signaling upon activation or differentiate properly due to dysregulation of transcription factor c-Maf [51], [52]. These findings demonstrate that miR-155 plays an important role in immune cell differentiation beginning in hematopoiesis and extending to B cell lymphoma and T lymphocyte differentiation. Taken with the identification of miR-155 as a target of Foxp3, further studies are needed to fully appreciate how miR-155 operates in cell intrinsic and extrinsic manners to determine physiological functions in Tregs and in other cellular environments.

The high expression of miR-155 induced by Foxp3 alludes to a potential relationship between miR-155 and Treg differentiation, function, or general maintenance. Indeed, high amounts of miR-155 are induced in tTreg precursors, and its continuous expression in Tregs is crucial for maintaining homeostasis while being dispensable for Treg differentiation or suppressive function [53]. Lu et al. have demonstrated a cell-intrinsic role for miR-155 in $Foxp3^{+}$ Tregs, where mice deficient in miR-155 exhibit reduced numbers of $CD4^{+}$ T cells and have a more pronounced defect in the Treg compartment. Through mixed bone marrow chimera experiments, this investigation revealed a cell intrinsic role for miR-155 in Treg homeostasis: miR-155 was shown to confer competitive fitness to Tregs by mediating the expression of suppressor of cytokine signaling-1 (SOCS1), which, consequently, alters sensitivity to limiting growth factor IL-

2 [53] [8]. Further investigation on how SOCS1 impacts immune cells through the miR-155 axis was accomplished by developing a SOCS1 knock in model, where the 3' UTR of SOCS1 transcript was mutated to ablate the miR-155 binding site [54]. While these experiments confirmed the mediation of SOCS1 by miR-155 to maintain Treg homeostasis in a lymphoreplete environment, the SOCS1 knock in mice did not demonstrate a reduction in Tregs at steady state. This finding was unexpected, as mice with the germ line deletion of miR-155 do show a deficit in the number of Tregs in the steady state condition. Collectively, these data suggest that there must exist miR-155 targets other than SOCS1 that are contributing to the observed phenotype in the Treg compartment of mice harboring the germ line deletion.

Because these experiments relied on germ line knock out mouse models where miR-155 was ablated in all cell types, other models are needed to confirm whether miR-155 regulates Treg development in a cell intrinsic or extrinsic fashion. To begin, continuing investigations on miR-155 in a Treg intrinsic manner requires specific ablation of miR-155 in $CD4^+$ T cells. On the other hand, we elected to study whether thymic epithelial cells (TECs) act as a cell extrinsic source of miR-155 in mediating tTreg development and homeostasis. Due to the indispensable role TECs play in determining the thymic microenvironment for the development of tTregs and their precursors, we generated mouse models where the expression of miR-155 is conditionally deleted in CD4 T cells (T-cKO) or in thymic epithelial cells (TEC-cKO) to further elucidate the mechanisms through which miR-155 acts to determine Treg development. Here, we identify a novel role for miR-155 in tTreg development where the proportion of tTregs is dependent on the expression of miR-155 as expressed by the thymic epithelia. Our results

suggest that miR-155 mediation of SMAD3 could be involved in limiting TGF-β signaling in mTECs to maintain the expression of CD80 and MHC II in the mature mTEC population and, consequently, a thymic microenvironment capable of generating tTregs. These results further imply that manipulation of thymic epithelial miRNAs may serve to fine-tune the thymic microenvironment in establishing a robust Treg population or even enhancing central tolerance.

RESULTS

Chapter 1: miR-155 promotes tTreg development in both T cell intrinsic and extrinsic manners

To determine whether the expression of miR-155 affects Treg development in a cell autonomous or nonautonomous fashion, we generated conditional knock out mice where miR-155 floxed mice (*miR-155*$^{fl/fl}$) were crossed to either CD4-Cre mice for a T cell conditional knockout (T-cKO) or FN1-Cre mice for a TEC conditional knockout (TEC-cKO) (Fig. 1). Isolation of CD4 single positive (CD4SP) T cells and thymic epithelial cells (TECs) from each mouse for analysis of miR-155 expression confirmed the specificity of the miRNA deletion in both the T cell and TEC compartments (Fig. 2).

Similar to what was reported in miR-155 germ line knockout mice (miR-155$^{-/-}$), the T-cKO (but not TEC-cKO) mice exhibited reduced CD4 T cell frequencies (Fig. 3A and B) [53]. In agreement with what was previously shown, the T-cKO mice demonstrated reduced Treg frequencies in both the thymus and periphery (Fig. 3C and D). Interestingly, the TEC-cKO mice also reflected this defect in the Treg compartment, but the reduction of Tregs in the periphery of these mice appeared to be less drastic as compared to the T-cKO mice (Fig. 3C and D). This supports the idea that the deletion of miR-155 in TECs is primarily affecting tTreg development while peripheral Tregs are less affected post-thymic emigration. Together, these results clearly demonstrate both T cell intrinsic and extrinsic axes for miR-155 in Treg development and homeostasis.

The results described in Chapter 1, in part, are currently being prepared for submission for publication, for which the thesis author serves as a leading author. (Warner, Lindsey; Dong, Jiayi; Lin, Ling-Li; Chen, Mei-Chi; Lu, Li-Fan).

Chapter 2: miR-155-Peli-1-c-Rel axis plays a minimal role in the development of tTregs

Since both miR-155 germ line knock out mice and the T-cKO mice demonstrate a defect in the Treg compartment, we sought to identify other T cell intrinsic targets of miR-155 apart from SOCS1 that may be responsible for regulating tTreg homeostasis. Recently, it has been shown that a novel miR-155-Peli-1-c-Rell pathway controls the generation and function of T follicular helper (Tfh) cells [56]. Here, miR-155 mediates the expression of Peli-1, an ubiquitin ligase that promotes the degradation of c-Rel. c-Rel has long been appreciated for its role in Treg development and homeostasis as it is required for the formation of the Foxp3 enhanceosome required for Treg differentiation [57]. This role of miR-155 in Tfh cell development has further been extended to include peripherally induced Tregs, but those derived from the thymus. Mice heterozygous for Peli-1 on a miR-155 deficient background show partial rescue of the Treg compartment in the spleen, mesenteric and peripheral lymph nodes compared to miR-155 deficient mice, while the ratio of thymus derived Tregs was unaffected (Liu, Kang et al., data not published). However, these studies utilized mouse models where miR-155 was ablated in all cells. Therefore, we were interested in whether the miR-155-Peli-1-c-Rel axis that acts in the periphery was also accountable for the decrease in tTregs we observed in the T-cKO mice. CD4 single positive thymocytes were isolated and then cultured with anti-CD3 and anti-CD28 for zero, 16, or 48 hours and then examined for the expression of Peli-1 and c-Rel (Fig. 4A). Thymocytes from both the wild type and T-cKO mice showed similar expression of Peli-1 and c-Rel across different time points, where the concentration of Peli-1 in the T-cKO did not correlate with a decrease in c-Rel (Fig. 4A, B, and C). Although we did observe slight upregulation of Peli-1 in the T-cKO at 16

hours, it was insufficient in promoting c-Rel degradation in thymocytes. Thus, the Peli-1-c-Rel axis is likely not conserved in thymocytes, as ablation of miR-155 in $CD4^+$ thymocytes showed no negative correlation between the presence Peli-1 and c-Rel.

To further confirm whether or not the Peli-1-c-Rel axis is important for thymic Treg development, we performed mixed bone marrow chimera experiments where bone marrow from wild type, *miR-155*$^{-/-}$, or *miR-155*$^{-/-}$ *Peli*$^{+/-}$ mice was mixed 1:1 with wild type $Ly5.1^+$ bone marrow and then reconstituted in lethally irradiated $Rag1^{-/-}$ mice that were analyzed at later time points (Fig. 5A). Peripheral blood lymphocytes analyzed at day 28 post-reconstitution did not show any change in the proportion of Tregs across all three groups (Fig. 5B and C). This was not entirely unexpected, as previous mixed bone marrow chimera experiments with *miR-155*$^{-/-}$ mice showed no defects in Treg homeostasis until analysis at much later time points [53]. However, after allowing enough time for total thymic reconstitution, the ratio of $Ly5.1^-$: $Ly5.1^+$ tTregs remained unchanged between the wild type, *miR-155*$^{-/-}$, and *miR-155*$^{-/-}$ *Peli*$^{+/-}$ mice, with the heterozygous condition unable to rescue the tTreg defect (Fig. 5B and D). Consistent with what has been previously demonstrated and what we have shown in the T-cKO model, the miR-155-Peli-1-c-Rel axis has a partial role in the periphery, but is unlikely implicated or necessary in the development of thymus derived Tregs

Chapter 3: Thymic epithelia miR-155 maintains mature mTECs through targeting TGF-β signaling

Previously, it has been reported that miR-155 is differentially expressed in the thymic epithelia, where mTECs highly express miR-155 in comparison to cTECs [55]. Similarly, we assessed the expression of miR-155 across various TEC populations in 5-6 week old WT mice. In agreement with earlier reports, we observed an upregulation of miR-155 in mTECs relative to cTECs (Fig. 6A). Because the TEC-cKO mice demonstrate a reduction in tTregs, we next sought to elucidate the molecular mechanisms through which miR-155 expression in the thymic epithelia determines tTreg development. Because the overall size and maturity of mTECs is known to impact tTreg development, we decided to assess the expression of CD80, MHC II, and AIRE in the mTEC population. These molecules are commonly used to characterize the stages of mTEC maturation, as adequate expression is required for providing co-stimulatory signals to developing thymocytes and for inducing the expression of TRAs to determine central tolerance [17]. Interestingly, upon further characterizing various sub-populations of mTECs, we have found that the more mature $CD80^{hi}$ $MHCII^{hi}$ mTECs express remarkably high levels of miR-155 when compared to their immature $CD80^{lo}$ MHC II^{lo} counterpart (Fig. 6B). To determine whether the Treg defect observed in the TEC-cKO mice was due to changes in mTEC composition, we isolated total TECs for analysis of cTECs, mTECs, and various mTEC subsets using methods similar to those previously described [58] [59]. Firstly, the proportions of cTECs and mTECs remained comparable between WT, T-cKO, and TEC-cKO mice (Fig. 7A and B). However, in depth characterization of the cell surface phenotype of mTECs showed a reduction in the frequency of mature $CD80^{hi}$MHC II^{hi} mTEC population in the TEC-cKO, with this defect

appearing to be further exaggerated in $CD80^{hi}$ population alone (Fig. 7C, D, and E). This implies that expression of miR-155 in the thymic epithelia is particularly crucial for maintaining the expression of co-stimulatory molecule CD80, as supported by the aforementioned miRNA profiling of WT mTECs where we confirmed miR-155 is highly upregulated in $CD80^{hi}$MHC II^{hi} mTECs (Fig. 6B). Quantification of the mean fluorescence intensity (MFI) of CD80 and MHC II in mTECs further substantiates this notion: a marked decrease in CD80 expression was seen in the TEC-cKO while the expression of MHC II actually remained relatively comparable between the WT, T-cKO, and TEC-cKO groups (Fig. 7G). These experiments identify a phenotypic consequence of miR-155 ablation in mTECs that likely accounts for the observed tTreg defect, and indicate a functional axis in mTECs through which miR-155 can impact the expression of CD80 and MHC II.

Because ablation of miR-155 in TECs results in a less mature mTEC population, we asked whether or not miR-155 impacts the more mature TRA expressing mTECs that depend on autoimmune regulator AIRE for development. Examination by flow cytometry did reveal a slight reduction in AIRE expression in the mTEC compartment, and MFI results similarly mimicked this downward trend in the expression of AIRE in the TEC-cKO mice (Fig. 7C, F, and G). While it is well known that AIRE is required early in life for establishing central tolerance, it cannot be said whether the mild decrease in $AIRE^{+}$ mTECs we report here results in the breakdown self-tolerance; previously characterized miR-$155^{-/-}$ mice do not develop spontaneous autoimmunity nor exhibit defects in Treg suppressor function [53] [60]. In spite of this, it remains unclear what level of AIRE expression is sufficient for generating a diverse tTreg repertoire physiologically adept at

controlling autoimmunity [21] [60]. These caveats considered, these data do affirm a role for miR-155 in maintaining the CD80^{hi} MHC II^{hi} mature mTEC population required for proper tTreg differentiation and development.

The growth and maturation of mTECs is largely mounted through RANK signaling, but can be fine-tuned by several negative feedback signaling cascades. TGF-β is a potent inhibitor of mesenchymal stem cell and stromal cell proliferation, and has recently been shown to limit the growth, differentiation, and function of the mature CD80^{hi}MHC II^{hi} mTEC population [22]. Attenuation of mTEC development through negative feedback on RANK has also been demonstrated by Spi-B-mediated OPG expression, where OPG competes with cytokine RANKL for interaction with RANK [23]. Both of these reports describe how loss of negative regulators TGF-β or OPG results in an increased thymic medulla and Treg compartment, where the extent of mTEC size and maturity has an impact on both Treg quantity and quality. Similar to what we show in our TEC-cKO mice, dysregulation of TGF-β signaling in the thymic epithelia results in reductions in the tTreg and CD80^{hi}MHC II^{hi} mTEC populations. Interestingly, miR-155 has previously been implicated at several levels of TGF-β signaling: activation of SMAD4 has been shown to induce miR-155 expression, and miR-155 targeting of SMAD2 regulates macrophage responses to TGF-β [61], [62]. Therefore, we next sought to determine whether miR-155 has a direct role in regulating mTEC development through the negative feedback effects of the Spi-B and/or canonical TGF-β signaling pathways. Prior analyses from high-throughput sequencing of RNA isolated from CD4^{+} T cells through cross-linking immunoprecipitation (HITS-CLIP) revealed a putative miR-155 binding site at the 3’ UTR of Spi-B as well as a binding site at the 3’ UTR of SMAD3, a

well-established downstream component of TGF-β signaling [63], [64]. Because this suggested possible miR-155 mediation of negative regulators that are known to limit mTEC development, we conducted luciferase reporter assays to establish whether miR-155 directly targets either Spi-B or SMAD3. miR-155 repression of Spi-B was unremarkable, suggesting that Spi-B is not regulated by miR-155 (Fig. 8A). On the other hand, co-transfection of miR-155 with the SMAD3 3'UTR resulted in diminished reporter activity compared to co-transfection of control miRNA, miR-150, whereas mutation of the miR-155 binding site in the SMAD3 3'UTR partially abolished this repression (Fig. 8B). Consistently, elevated amount of SMAD3 was found in mature $CD80^{hi}MHCII^{hi}$ mTEC population isolated from TEC-cKO mice. And while we did not observe any statistically significant differences in our pilot experiment, the loss of miR-155 in TECs also resulted in a detectable increase in the mRNA level of Myc, a well-established TGF-β target (Fig. 8C). While further studies are required to confirm our preliminary observations, nevertheless, our data point to a miR-155-SMAD3 axis that modifies TGF-β signaling in the thymic medulla to determine the quantity of tTregs generated during development.

The results described in Chapter 3, in part, are currently being prepared for submission for publication, for which the thesis author serves as a leading author. (Warner, Lindsey; Dong, Jiayi; Lin, Ling-Li; Chen, Mei-Chi; Lu, Li-Fan).

DISCUSSION

While the regulatory functions of microRNAs are well appreciated and implemented in virtually all biological processes, further context and cell-specific investigations of already well characterized miRNAs is required to clarify their distinct roles in a variety of environments. Here, we identify a new cell-extrinsic role for miR-155 in fine-tuning mTEC maturity to determine the thymic microenvironment for the generation of Tregs. Our data propose a miR-155-SMAD3 axis in TGF-β signaling where dysregulation of miR-155 results in the increased expression of TGF-β effector molecules and, consequently, a diminished $CD80^{hi}MHCII^{hi}$ mTEC population. It is likely this defect results from impaired mTEC proliferation, as previous studies have shown TGF-β to be have a specific and potent anti-proliferative effect on the thymic medulla [22]. However, because TGF-β has both proapoptotic and anti-proliferative effects, further investigation is required to determine the extent of miR-155 mediation of TGF-β signaling and, thus, mTEC proliferation and programmed cell death [65].

Our follow up studies on the T cell intrinsic effects of miR-155 imply that the miR-155-Peli-1-c-Rel axis important for T cell development in the periphery but is not conserved in thymocytes. For this reason, additional studies are needed to identify miR-155 targets apart from SOCS1 that may be involved in Treg development and homeostasis. On the other hand, cell extrinsic expression of miR-155 in the thymic epithelia revealed an important relationship between miRNA expression in mTECs and the development of tTregs. The quantity of tTregs generated is dependent on mTECs and

the degree to which they express antigen presenting and co-stimulatory molecules. We report that deletion of miR-155 in the thymic epithelia results in a less mature mTEC population that cannot support normal Treg development. While this decrease in $CD80^{hi}MHC\ II^{hi}$ mTECs impacts tTreg quantity, whether it also affects the quality of the Treg repertoire remains to be elucidated. Studies disrupting canonical miRNA assembly complexes in the thymic epithelia have previous illustrated a role for miRNAs in negative selection; deletion of miRNA processor DGCR8 in TECs results in an mTEC population with impaired AIRE expression, which inherently limits the tTreg repertoire and results in incomplete central tolerance [66]. Certainly, these previous reports have demonstrated that miRNAs play an important role in the homeostasis of mature $AIRE^{+}$ mTECs, but our data do not directly support a role for miR-155 in determining the expression of TRAs in the thymic medulla. While we observed a slightly diminished number of $AIRE^{+}$ mTECs in the absence of thymic epithelial miR-155, TEC-cKO mice showed no signs of autoimmune disease. However, aging studies may offer a more complete answer to whether or not miR-155 has a role in establishing central tolerance, as the mice in our investigations were assessed at ages too young to observe spontaneous onset of autoimmunity.

While the responsibility of miRNAs as global regulators of immune cell development and homeostasis continue to be substantiated, the individual miRNAs that modulate gene expression in TECs remain, by and large, unknown. Here, we identify a non-redundant role for thymic epithelial miR-155 in mediating SMAD3 and other downstream components of TGF-β signaling to shape the thymic microenvironment for tTreg development. These findings underscore the importance of understanding cell-

specific miRNA functions in the thymic medulla and encourage further examination into how individual miRNAs contribute to Treg development and central tolerance. The mechanistic insights presented here point to an additional potential for miRNA therapeutics in supporting TEC function to boost Tregs or self-tolerance.

MATERIALS & METHODS

Mice

Mice containing T-cell specific deletion and thymic epithelial cell-specific deletion of miR-155 were generated by breeding miR-155$^{fl/fl}$ mice (Ryan O'Connell, University of Utah) to CD4-Cre mice and FN1-Cre mice, respectively. miR-155$^{-/-}$ and miR-155$^{-/-}$ Peli-1$^{-/+}$ mice were generated as previously described (reference Changchun lab). All mice were maintained and handled in compliance with the Institutional Animal Care and Use Guidelines of the University of California, San Diego and the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

miRNA Expression Profiling & Quantitative PCR Analysis

CD4SP T-cells were sequestered from CD4-Cre X miR-155$^{fl/fl}$ mice via Dynabead CD4 Positive Selection Kit (Invitrogen). EpCAM^{+}CD45^{-} thymic epithelial cells from FN1-Cre X miR-155 fl/fl mice were sorted by FACS Aria. Isolated cells were subjected to RNA isolation using miRNeasy kit (QIAGEN). Isolated RNA was subjected to miRNA expression profiling analysis of miR-155 by qPCR and Taqman (Thermo Fisher Scientific) stem-loop real-time RT-PCR, as previously demonstrated (Lu et al., 2010). Primers are as follows:

Bone Marrow Reconstitution

T-cell-depleted bone marrow cells from wild type mice expressing the congenic marker CD45.1 were mixed with bone marrow from wild type, miR-155$^{-/-}$, or miR-155$^{-/-}$ Peli-1$^{-/+}$ mice expressing the congenic marker CD45.2 in equal ratios and then injected into lethally irradiated (950 rads) wild type CD45.1 mice (Lu et al., 2009).

Ex Vivo Phenotyping & Flow Cytometry

Single-cell suspensions of thymus, spleen, and PLN were prepared by slide mechanical disruption. To isolate thymic epithelial cells, thymus was cut and washed in RPMI-1640 (plain), followed by enzymatic digestion (0.10% DNase I; 0.125% Collagenase, 0.125% Collagenase/Dispase; Roche). Cells were washed in PBS and then stained in FACS buffer (5% FBS in PBS) with viability dye eFluor 780 or 450 (eBioscience). Antibodies for extracellular staining include CD4, CD8a, CD44, CD62L, CD45, EpCAM, Ly 51, UEA-1, MHC II, CD80, and Streptavidin (eBioscience; Biolgenend). Intracellular staining for Foxp3, Ki67, and AIRE was completed after fixation and permeabilization according to manufacturer protocol (Tonbo). Cells were fixed in 2% paraformaldehyde prior to analysis by flow cytometry. Data was analyzed by FACS Diva and FloJo software (BDBiosciences; Tree Star).

Immunoblotting

CD4 single positive T cells were isolated by dynabead CD4 positive selection kit (Invitrogen) and cultured in 24-well plates coated with 2 μg/mL of α-CD3 and 5μg/mL of α-CD28 (1 x 10^6 cells/mL). Cells were harvested 16, 24, or 48 hours later and lysed with RIPA buffer containing 1mM PMSF for 20 minutes. Cell lysates were partitioned by SDS-PAGE and then transferred onto PVDF membranes (Bio-Rad Laboratories). Antibodies for c-Rel, Peli-1, and β-Actin (Sigma-Aldrich) were used to visualize proteins. Protein concentration was quantified via ImageJ (National Institutes of Health).

Luciferase Reporter Assay

The 3′ UTR of SMAD3 or SOCS1 were cloned into psiCheck2 (Promega). miR-155 and miR-150 sequences were respectively cloned into pMDH-PGK-EGFP, as previously described (Lu et al., 2009). 1 day prior to transfection, HEK293T cells were plated at 6.5 × 10^4 cells per well on a 24-well plate. PsiCheck2 bearing SMAD3 or SOCS1 3′ UTRs were transfected to HEK293T cells with control empty vector or miR-155 and mIR-150 expressing plasmids using Fugene 6 (Promega). Luciferase activities were measured 24 hours post-transfection by Dual-Luciferase Reporter assay system (Promega) according to the manufacturer's protocol.

Statistical Analyses

Unpaired Student's t test was done on all reported data using Prism software (GraphPad) (*P < 0.05; **P < 0.01; ***P < 0.001).

FIGURES & FIGURE LEGENDS

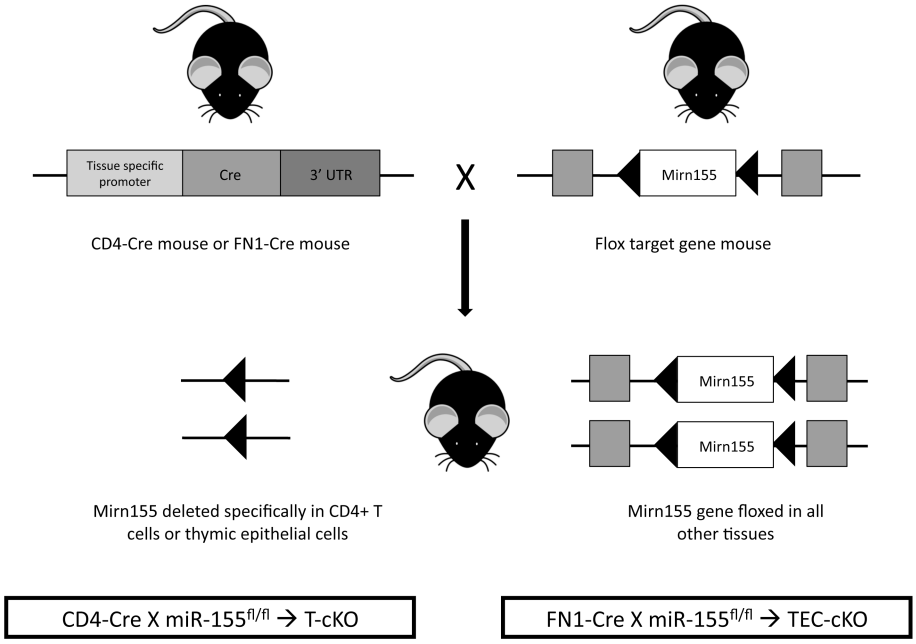

Figure 1. Schematic of the generation of TEC conditional knockout (TEC-cKO) and CD4 T cell conditional knockout mice (T-cKO). Under the control of tissue specific promoters Foxn1 (FN1) and CD4, Cre-mediated recombination results in the specific deletion of miR-155 in the thymic epithelial cells (TECs) and $CD4^+$ T cells, respectively.

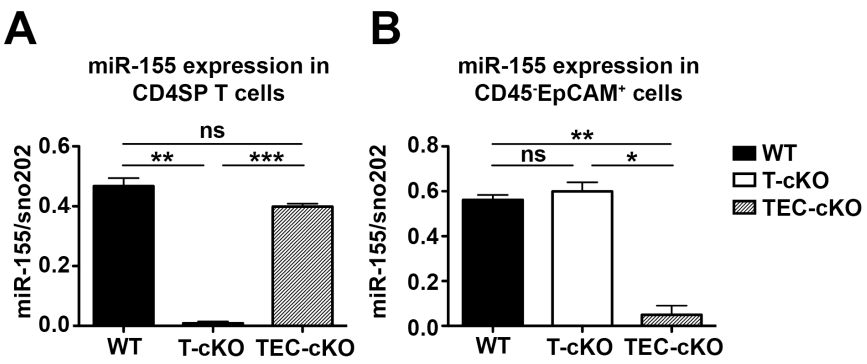

Figure 2. Expression of miR-155 in TECs and CD4SP T cells. qPCR analysis for the expression of miR-155 in TECs and CD4SP T cells. Total thymocytes were sorted for $CD45^{-}EpCAM^{+}$ TECs and $CD4^{+}CD8^{-}$ CD4SP T cells. Data are representative of three independent experiments (n = 4).

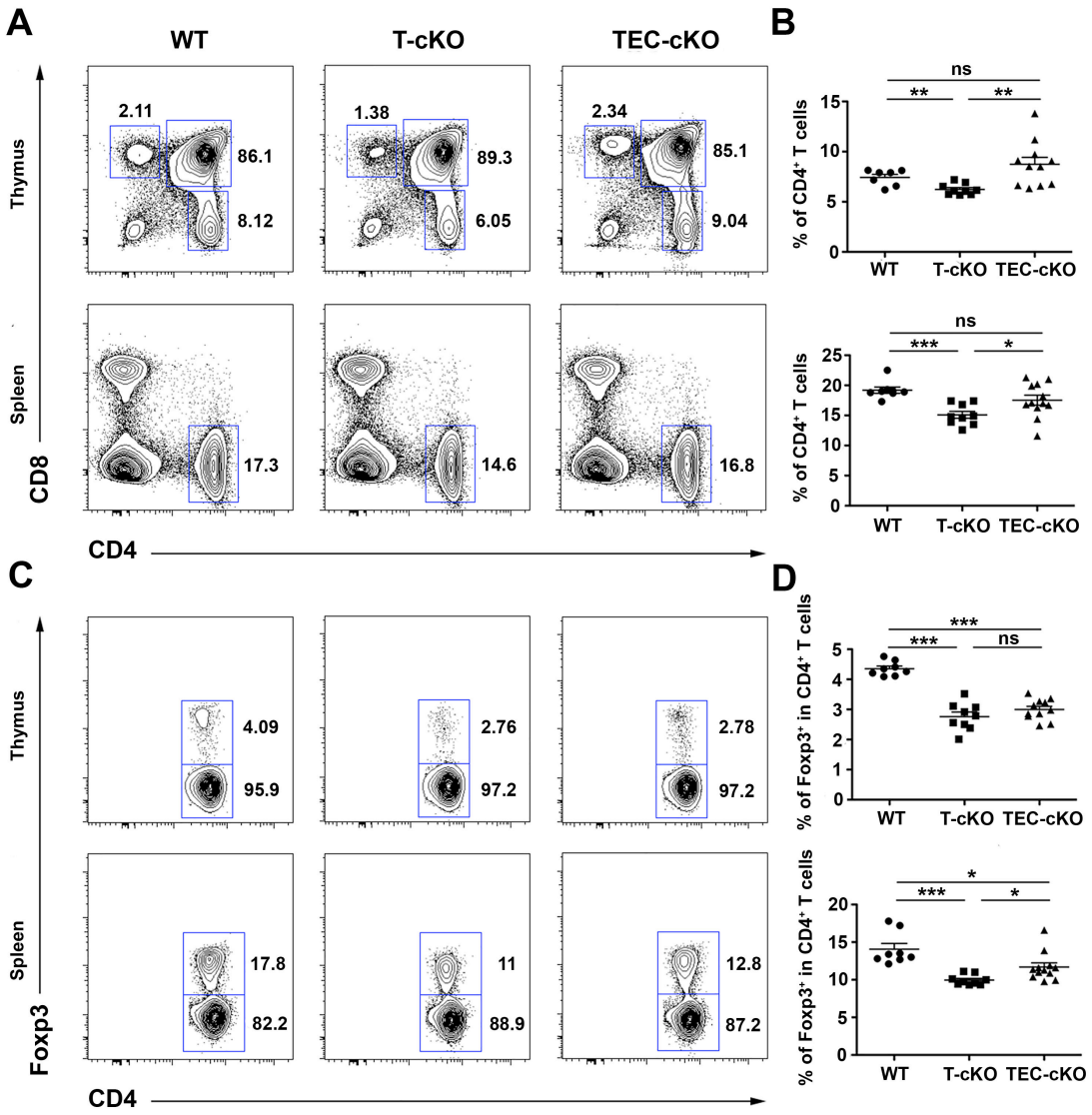

Figure 3. Both T-cKO and TEC-cKO mice demonstrate a reduction in Tregs. Analysis of CD4 T cells and Foxp3^{+} Tregs in the thymus and spleen of 5-6 week of WT, T-cKO, and TEC-cKO mice. Frequency comparison of total CD4 T cells are shown for the thymus and spleen (A and B). The frequency of Foxp3^{+} Tregs are similarly shown for thymus and spleen (C and D). Data are representative of 8 independent experiments (n = 4-8).

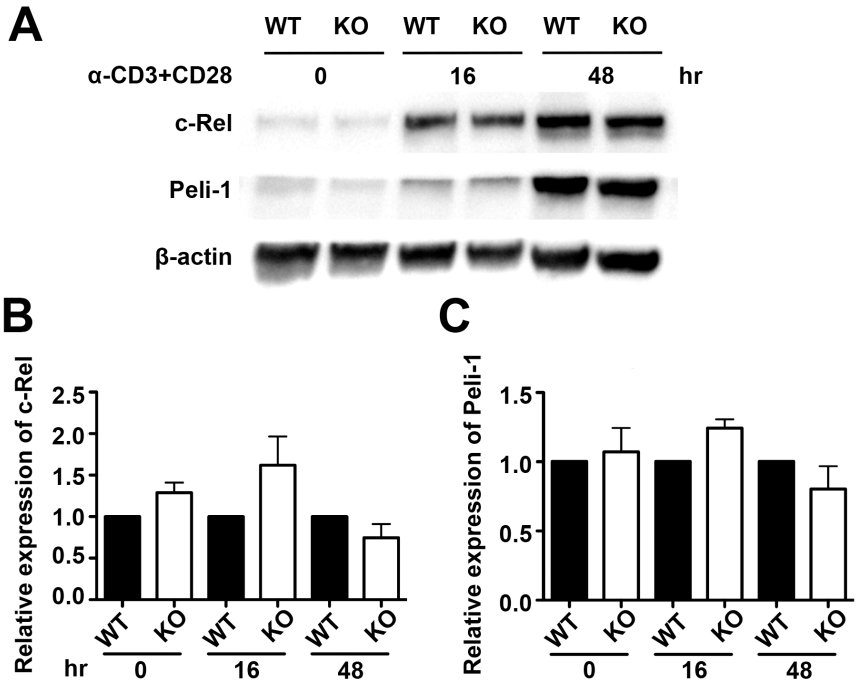

Figure 4. Immunoblot analysis of c-Rel and Peli-1 expression in CD4SP T cells. CD4SP T cells were cultured for 0, 16, or 48 hours and cell lysates were collected for SDS-Page (A). Densitometric c-Rel (B) and Peli-1 (C) expression was normalized to β-actin and reported as n-fold change in expression based on each corresponding WT. 250K cells were loaded per well. Data are representative of 4 experiments (n = 4).

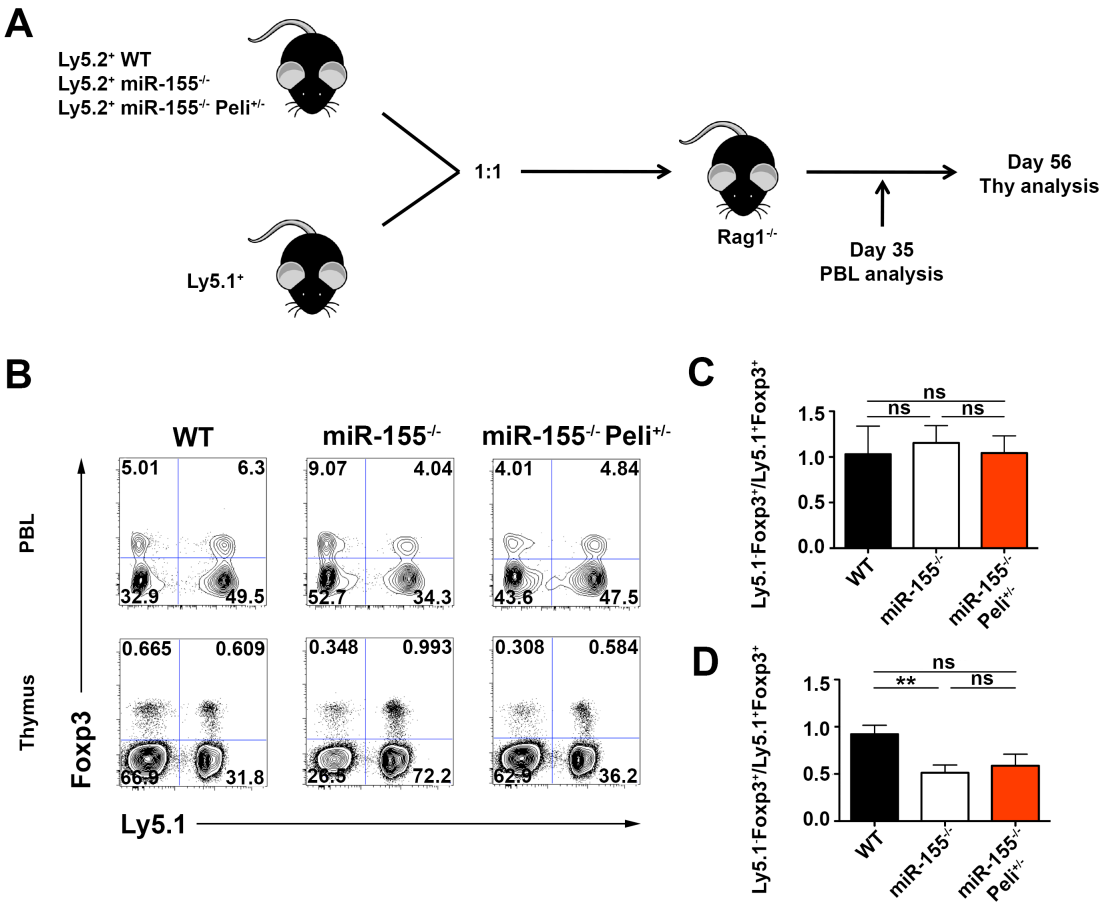

Figure 5. Ratios of Ly5.1$^-$Foxp3$^+$ and Ly5.1$^+$Foxp3$^+$ cells in BM chimeras. BM from Ly5.2$^+$ WT, miR-155$^{-/-}$, or miR-155$^{-/-}$Peli$^{+/-}$ mice was mixed 1:1 with BM from WT Ly5.1$^+$ and injected into lethally irradiated Rag1$^{-/-}$ mice (A). Flow cytometry analysis of Tregs from peripheral blood lymphocytes (PBLs) and the thymus at 4 weeks and 8 weeks post-reconstitution, respectively (B). Ratios of Ly.1$^-$:Ly5.1$^+$ Tregs for PBLs and thymus are reported for WT, miR-155 KO, and miR-155 KO Peli +/- mice in PBL (C) and thymus (D). Data are representative of 2 independent experiments.

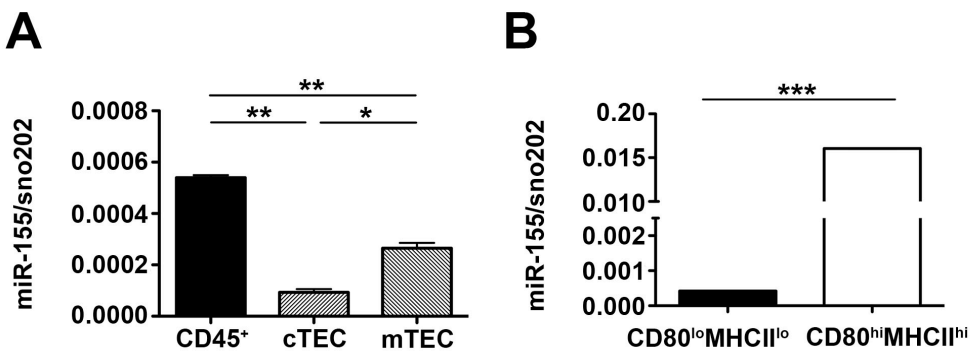

Figure 6. Distinct expression patterns of miR-155 in hematopoietic cells versus thymic epithelial cells. qPCR analysis for the expression of miR-150 and miR-155 in different thymocytes subsets. Total thymocytes were sorted for $CD45^{+}EPCAM^{-}$ hematopoietic cells, $CD45^{-}EpCAM^{+}Ly51^{+}UEA\text{-}1^{-}$ cTECs, $CD45^{-}EpCAM^{+}Ly51^{-}UEA\text{-}1^{+}$ mTECs (A). Similarly, total thymocytes were sorted for $CD80^{lo}$ MHC II^{lo} and $CD80^{hi}$ MHC II^{hi} within the $UEA\text{-}1^{+}$ mTEC population (B). Data are representative of 2 independent experiments (n = 3-4).

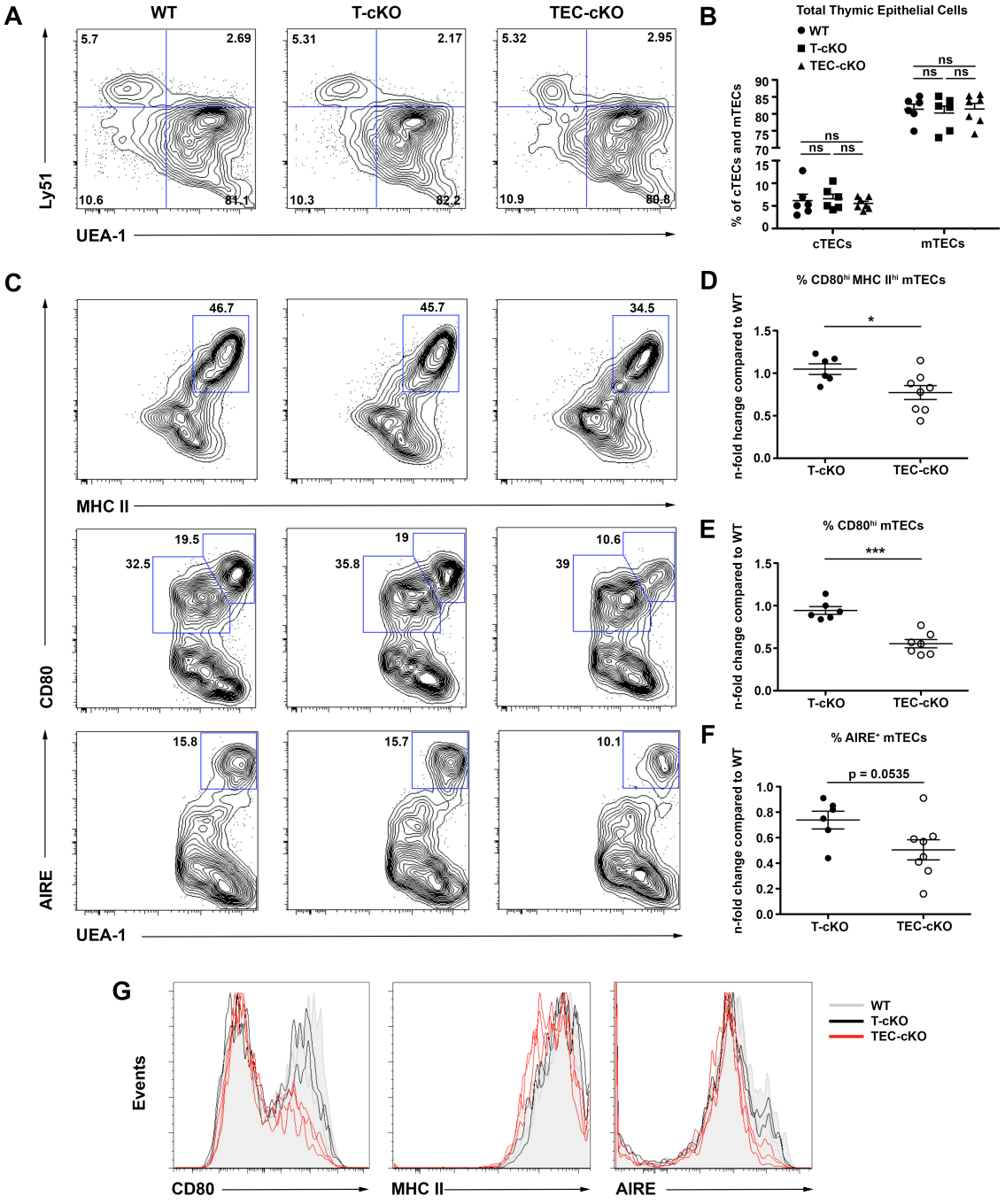

Figure 7. Deletion of miR-155 in the thymic epithelia impacts the maturity of various mTEC populations. Flow-cytometric analysis of cTECs and various mTEC populations in 5-6 week old WT, T-cKO, and TEC-cKO mice. Ly51^{+}UEA-1^{-} cTECs and Ly51^{-}UEA-1^{+} mTECs were characterized by flow cytometry (A) and total mTEC frequency was reported to show no change across experimental groups (B). mTECs were further characterized for their expression of CD80, MHC II, and AIRE (C) to draw distinctions between mature and immature mTECs. The n-fold change of percent of CD80^{hi}MHC II^{hi} (D), CD80^{hi} alone (E), and AIRE^{+} (F) mTECs from the T-cKO and TEC-cKO mice are normalized to WT frequencies. Mean Fluorescence Intensity for CD80, MHC II, and AIRE are shown for all experimental groups (G). Data are representative of 5 independent experiments (n = 4-7).

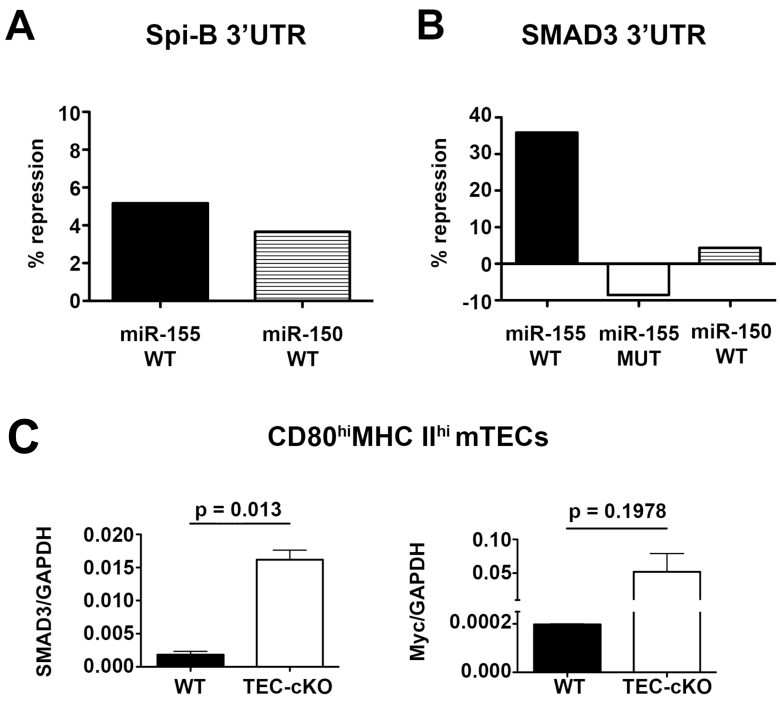

Figure 8. miR-155 targets SMAD3 to fine-tune TGF-B signaling. Percent repression of the Spi-B 3'UTR (A) and SMAD3 3'UTR (B) as determined during luciferase reporter assay on HEK293T cells transfected with either with miR-155 or miR-150. Data are representative of 3 independent experiments. Relative expression of SMAD3 and Myc mRNA transcripts in the mature $CD80^{hi}MHC\ II^{hi}$ mTECs from WT and TEC-cKO mice (C). Data are representative of a single preliminary study.

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