UNIVERSITY OF CALIFORNIA SAN DIEGO

The Role of the MicroRNA-23-27-24 Family in Intratumoral Regulatory T-cells

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by

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TABLE OF CONTENTS

LIST OF FIGURES

ACKNOWLEDGEMENTS

Chapter 1, in part, includes unpublished data from Cheng-Jang Wu. The thesis author is the primary author of this thesis.

Chapter 2, in part, includes unpublished data from Cheng-Jang Wu. The thesis author is the primary author of this thesis.

ABSTRACT OF THE THESIS

Role of the MicroRNA-23-27-24 Family in Intratumoral Regulatory T-cells

by

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MicroRNAs (miRNAs) are small non-coding RNA molecules that regulate the transcriptome by degrading or suppressing the translation of target mRNAs. Loss of the microRNA network in regulatory T-cells (Tregs) leads to the development of systemic autoimmunity and inflammation due to loss of immunological control. Previously our lab discovered critical roles of the miR-23-27-24 (miR-23) family in controlling the differentiation and function of follicular

helper and effector T-cells. To explore the functions of the entire miR-23 family in Tregs, we utilized a transplantable tumor model. We found that loss of the miR-23 family in Tregs impeded tumor growth and reduced Treg frequency. However, there was no apparent increase in the effector functions of antitumor $CD8^+$ T-cells because miR-23 deficient Tregs were more suppressive, compensating for reduced intratumoral Tregs. This increase in suppression is potentially attributed to an increase in IL-10, a target of miR-27. Further profiling of the immune cell landscape found an increase in Natural Killer (NK) cells, which have been shown to be activated by IL-10. The transcription factor TCF1, a target of miR-24, was found to be increased in miR-23 family deficient intratumoral Tregs, leading to a loss of $TCF1^-$ effector Tregs. RNA-sequencing found that loss of the miR-23 family in intratumoral Tregs led to a reduction in IL2-STAT5 signaling. Collectively, our work demonstrates that loss of the miR-23 family in Tregs leads to enhanced suppression, potentially due to increased IL-10, loss of $TCF1^-$ Tregs, and impaired proliferative capacity.

INTRODUCTION

Chapter 1: Regulatory T-cell Development and Functions in Periphery

Regulatory T-cells (Tregs) are a subset of $CD4^+$ T-cells that express the X-chromosome linked lineage determining transcription factor Forkhead Box P3 (Foxp3), which is essential for the development of the suppressive program of Tregs. Loss of Foxp3 in mice leads to the development of severe autoimmunity, multi-organ inflammation, and early death in mice **(**Brunkow et al., 2001; Lin et al., 2005**)**. This highlights the critical role that Tregs play in maintaining peripheral tolerance and in controlling overt immune responses. Similarly in humans, mutations in the Foxp3 gene locus or impairment of Treg functions can lead to the development of immunodysregulation, polyendocrinopathy, enteropathy, and X-linked syndrome (IPEX) **(**Bennett et al., 2001**)**. Even short-term deletion of Tregs using a Foxp3-DTR mouse model leads to the onset of autoimmune pathology in adult mice treated with diphtheria toxin (DT) **(**Kim et al., 2007**)**. These studies indicate that Tregs are needed to maintain a fine balance to allow the immune system to mediate its protective functions while preventing the development of autoimmunity.

Beginning their development in the bone marrow, lymphocyte progenitor cells migrate to the thymus to undergo maturation and develop into functional thymus-derived lymphocytes (T-cells). In the thymus, T-cells develop a functional T-cell receptor (TCR) that can recognize processed antigens presented on major histocompatibility complexes (MHCs) expressed on antigen-presenting cells (Zinkernagel & Doherty, 1974). To develop a functional TCR, double negative thymocytes ($CD4^-CD8^-$) initiate VDJ recombination to develop the TCR-β subunit, mediated by gene splicing enzymes RAG1/2 (Mombaerts et al., 1992; Oettinger et al., 1990). Upon generation of the TCR-β subunit, a pre-TCRα subunit associates with TCR-β, forming a pre-TCR complex that mediates intracellular signaling to initiate genetic rearrangement to generate the

TCR-α subunit and to upregulate CD4 and CD8 (Winandy et al., 1999). The newly generated TCR, expressed on double positive ($CD4^+CD8^+$) cells, interacts with MHC class I or II expressed by cortical thymic epithelial cells (cTECs) in the thymic cortex (Hogquist et al., 1994). Thymocytes with functional TCRs receive survival signals to continue maturation while those without functional TCRs undergo death by neglect. After positive selection, depending on whether they interact with MHC class I or II, they will commit to the $CD4^-CD8^+$ (CD8SP) or $CD4^+CD8^-$ (CD4SP) lineages, respectively (Chopp et al., 2023). Migrating to the thymic medulla, these single positive thymocytes interact with MHC class I or II expressed on medullary thymic epithelial cells (mTECs). These mTECs express the transcription factor AIRE, which allows for the presentation of tissue-specific self-antigens. CD4SP or CD8SP cells with TCRs that interact with these self-peptide-MHC complexes with high affinity undergo negative selection, inducing apoptosis to prevent self-reactive T-cells from entering the periphery (Liston et al., 2003). However, CD4SP cells that receive signals of moderate strength will express the transcription factor Foxp3, committing these self-reactive CD4SP T-cells to become thymic Tregs (Jordan et al., 2001; Malchow et al., 2016). Upon maturation, these conventional T-cells (Tconvs) and Tregs move into the circulation and patrol secondary lymphoid organs (SLOs) and peripheral tissues. Beyond thymic development, Tregs can also be induced in the peripheral tissues such as the lung, skin, and intestine and can provide tolerance against non-self-antigens (Apostolou & Boehmer, 2004). These developmental processes allow for self-antigen and non-self-antigen recognizing Tregs to provide immunological tolerance.

To maintain peripheral tolerance, Tregs can control immune responses by the expression of membrane-bound molecules/receptors and secrete immunosuppressive molecules through contact-dependent mechanisms. Tregs can mediate the direct killing of effector cells by releasing

the contents of intracellular granules that contain Granzyme-B and perforin, which initiates an apoptotic cascade in target cells **(**Gondek et al., 2005**)**. To prevent the activation of naïve T-cells, Tregs can also disrupt the contact of Tconvs and APCs, preventing necessary costimulatory signals from being transmitted to Tconvs **(**Tadokoro et al., 2006**)**. Another method to limit the costimulation of Tconvs involves the surface expression of CTLA-4 on Tregs. CTLA-4, which binds with high affinity to CD80 and CD86 costimulatory ligands, provides a mechanism of immune control by depriving conventional T-cells of costimulation by CD80/86 presenting APCs **(**Takahashi et al., 2000; Wing et al., 2008**)**.

Another method of immune regulation involves the consumption of key proliferation-inducing cytokines, the generation of suppressive molecules, and the release of inhibitory cytokines. Tregs were initially defined by high expression of the IL-2Rα (CD25), which forms a high affinity IL-2 receptor with IL-2Rβ and IL-2Rγ **(**Takahashi et al., 1998**)**. IL-2 is a critical cytokine that supports the proliferation of conventional T-cells upon activation, allowing for their rapid expansion. By expressing high levels of CD25, Tregs can consume available IL-2 in the microenvironment, preventing the expansion of conventional T-cells **(**Barthlott et al., 2005; Thornton & Shevach., 1998**)**. Surface expression of ectoenzymes CD39 and CD73 catalyzes the generation of adenosine from ATP. Generated adenosine can bind to A_2AR receptors expressed on conventional T-cells, mediating suppression **(**Deaglio et al., 2007**)**. Immunosuppressive cytokines such as TGF-β, which can be membrane-bound or secreted, and IL-10 have been demonstrated to play important roles in the induction and maintenance of regulatory T-cells as well as suppressing the effector functions of conventional T-cells in peripheral tissues **(**Asseman et al., 1999; Konkel et al., 2017; Rubtsov et al., 2008**)**. Through these various mechanisms, Tregs

function to maintain peripheral tolerance and control immune responses, preventing severe immunopathology and autoimmunity.

Chapter 2: Regulatory T-cells in the Development of Cancer

Cancer is a heterogeneous disease defined by the uncontrollable growth of cells due to the accumulation of mutations and aberrant chromosomal rearrangements. Various intracellular mechanisms exist to prevent the development of cancer by inducing apoptosis in mutated cells. However, sometimes these cancerous cells can escape programmed cell death, requiring the immune system to eliminate them before they develop into solid tumors. Natural Killer (NK) cells and $CD8^+$ T-cells patrol the body, identify cancerous cells, and induce apoptosis to prevent cancer development. Mutations in cancerous cells can lead to alterations in the amino acid sequence of endogenous proteins or induce the expression of tissue-restricted antigens (TRAs), allowing $CD8^+$ T-cells to target these cells for apoptosis by recognizing neo-antigens and TRAs presented on MHC-class I molecules (Bruggen et al., 1991; Mandelboim et al., 1994). To escape the anti-tumor immune responses of $CD8^+$ T-cells, cancerous cells can be selected over time to downregulate MHC-class I, preventing CD8-mediated apoptosis (Dhatchinamoorthy & Colbert., 2021). However, NK cells, which are inhibited by MHC-class I molecules, can recognize these MHC-class-I deficient cells in an antigen-independent manner to mediate cell lysis (Daniels et al., 1994; Karre et al., 1986; Liao et al., 1991).

To evade the immune system, tumors promote a microenvironment that can suppress anti-tumor responses. Alterations in intratumoral metabolites, acidity, cytokines, and immune cell profiles can promote an immunosuppressive environment. Expression of various chemokine receptors, such as CCR5, CCR6, CCR8, and CXCR3 on Tregs allows recruitment to the tumor by migrating along chemokine gradients (Sarkar et al., 2021). Once inside the tumor, Tregs can impede anti-tumor responses by lysing effector cells, generating immune-suppressive metabolites, consuming available IL-2, and secreting immunosuppressive cytokines (TGF-β and IL-10) to

maintain a suppressive tumor microenvironment (TME). Intratumoral Tregs can also support the development of vasculature (angiogenesis) by secreting endothelial growth factors such as amphiregulin (Areg), which binds to the endothelial growth factor receptor (EGFR) expressed on tumor cells to promote tumor growth (Arpaia et al., 2015; Sun et al., 2023). Areg can also bind to EGFR expressed on Tregs, promoting their suppressive capacity (Zaiss et al., 2013). Due to the deficiency of available glucose in the TME, Tregs rely on fatty acid oxidation (FAO) and oxidative phosphorylation to fuel their metabolic needs, allowing them to enhance their suppressive capacity and survive within tumors (Gerriets et al., 2016). Due to the high concentration of lactate within the TME, Foxp3 also reprograms intratumoral Tregs to utilize lactate to fuel oxidative phosphorylation (Angelin et al., 2017). These metabolic changes provide Tregs with the means to survive an otherwise harsh and nutrient-deficient microenvironment.

Due to Tregs being enriched within tumors and serving a critical role in promoting the suppression of anti-tumor responses, attempts have been made to modulate or deplete Tregs to treat cancer. Administration of CD25-specific depleting antibodies in tumor bearing mice has demonstrated that the loss of Tregs leads to a reduction in tumor growth (Golgher et al., 2002; Jones et al., 2002). However, deletion of Tregs can lead to the induction of autoimmune symptoms, limiting the therapeutic application of complete Treg deletion (Kim et al., 2007; Wei et al., 2005). To overcome this, targeting specific Treg subsets within the tumor or modulating their functions can complement existing therapeutics to improve clinical outcomes and prevent the onset of autoimmunity.

Chapter 3: MicroRNAs in Regulatory T-cells and the miR-23 Family

MicroRNAs are small non-coding RNA molecules that are around 23 nucleotides in length and function by degrading or suppressing the translation of target mRNAs, allowing for dynamic regulation of the transcriptome. These regulatory molecules were initially discovered in *C. elegens* and were further characterized in mammals and plants, with loss of microRNAs being associated with various diseases in animals (Hutvagner & Simard., 2008; Lau et al., 2001). Beginning with transcription of a primary microRNA (pri-miRNA) transcript by RNA Polymerase-II, this pri-miRNA gets processed by the RNase-III enzyme Drosha, in association with two subunits of DGRC8, to cleave the pri-miRNA to form a hairpin structure called the pre-miRNA (Landthaler et al., 2004; Lee et al., 2004). Exportin-5 binds and transports the pre-miRNA from the nucleus and into the cytoplasm for further processing (Yi et al., 2003). Once in the cytoplasm, the RNase-III enzyme Dicer, processes the pre-miRNA to form a dsRNA duplex (miRNA:miRNA*) that consists of a mature strand and a passenger strand. Argonaute proteins (AGO), specifically AGO2, associate with other proteins and the mature miRNA strand to form the RNA-induced silencing complex (RISC) (Pratt & MacRae., 2009). The RISC complex mediates degradation or suppression of translation by using the microRNA seed sequence to target binding sites within the 3' untranslated region (3'-UTR) of the target mRNA transcript, allowing for dynamic regulation of the transcriptome.

MicroRNAs serve critical roles in the function and differentiation of T-cells. Loss of the microRNA network by deleting Drosha in T-cells leads to dysfunctional development and alterations in T-cell subsets, driving inflammation and T-cell activation (Chong et al., 2008). Furthermore, specific loss of the microRNA network in Tregs leads to a phenotype strikingly similar to that of Foxp3 knockout mice, with the development of severe inflammation, immune

cell invasion in tissues, autoimmunity, and early lethality (Zhou et al., 2008). These studies highlight the importance of microRNAs in controlling T-cell and Treg function and homeostasis.

The microRNA-23-27-24 family consists of two clusters, miR-23a and miR-23b clusters, which are located on chromosomes 8 and 13 in mice, respectively. Previously our lab investigated the role of the miR-23-27-24 family in T-follicular helper (TFH) and effector T-cells. Loss of miR-23 family in T-cells led to enhanced TFH responses upon viral infection by increasing the levels of TOX, a target of miR-23 and miR-27 (Wu et al., 2019). In effector cells, we also found that the miR-23 family regulated the differentiation of different T-helper subsets (Cho et al., 2016). We also found that overexpression of miR-24 alters effector cell responses by targeting TCF1, leading to an increase in IFNγ and IL-17-expressing T-cells (Cho et al., 2017). In Tregs, we had previously demonstrated that overexpression of miR-27 leads to impaired Treg function by downregulating effector cytokines and transcription factors necessary for Treg development (Cruz et al., 2017). Due to the role of the miR-23 family in various T-cell subsets, we wondered how the loss of the miR-23 family could influence regulatory T-cells.

In this work, we investigate how the entire miR-23 family regulates the function of Tregs in a tumor model. Using a Foxp3-YFP-Cre mouse system to delete the miR-23 family in Tregs, we found a reduction in tumor growth in various tumor models. Analysis of immune cell populations within the tumor found an overall reduction in the frequency of Tregs. However, there was no increase in the effector responses of $CD8^+$ T-cells, a critical immune cell subset for antitumor responses. Using in vitro suppression assays, we found that while miR-23 family deficient Tregs had no difference in suppressive capacity at basal state, intratumoral miR-23 deficient Tregs were more suppressive than their wild-type counterparts. This enhanced suppression could partially be explained by an increase in immunosuppressive cytokine IL-10,

which is a target of miR-27. Furthermore, flow cytometric analysis found an increase in the frequency of NK cells, another critical anti-tumor immune cell population. IL-10 has been previously demonstrated to prevent activation-induced cell death (AICD) and increase the effector functions of NK cells. RNA-sequencing of intratumoral Tregs found that there was a reduction in IL2-STAT5 signaling in miR-23 family deficient Tregs, which was reflected by a reduction in Ki67 expression in $CD25^+$ Tregs lacking the miR-23 family. RNA-seq also found a reduction in TGF-β signaling and an increase in the transcription factor TCF1, which is a target of miR-24. Further analysis found an increase in $TCF1^+$ Tregs and TCF1 protein per cell. Surprisingly, the reduction of intratumoral Tregs deficient in the miR-23 family was accounted for by a loss of $TCF1^-$ Tregs, suggesting TCF1 is playing a key role in the homeostasis of Tregs within the tumor. Overall, we uncovered key nodes that the miR-23 family targets to modulate the suppressive capacity and homeostasis of intratumoral Tregs.

RESULTS

Chapter 1: Loss of miR-23 Clusters in Tregs Leads to Minimal Alteration to the Immune Cell Landscape at Steady State

Our previous studies in different T-cell subsets demonstrated a crucial role for the miR-23 family in regulating the differentiation and functions of T-cells by targeting critical transcription factors and effector molecules. We demonstrated that overexpression of miR-27 in Tregs led to impaired development and a reduction in suppressive capacity (Cruz et al., 2017). To further explore the roles of the entire miR-23 family in regulating Treg homeostasis and functions we generated mice with Treg-specific deletion of the miR-23a/b clusters (DKO) by crossing Foxp3-YFP-Cre (WT) mice with miR-23a/b cluster floxed mice. Leveraging this mouse model, we first sought to determine the functions of the miR-23 family at steady state.

To determine the functional consequences of miR-23 family ablation in Tregs, we examined the spleen, small intestine, and lung of WT and DKO mice. In the spleen, we found that the frequency of Tregs and expression of Foxp3 showed no significant difference between WT and DKO mice (Fig. 1A & B). We also found that there was no alteration in the frequencies of $Ki67^+$ Tregs and $IFN\gamma^+$ Tconvs; however, there was a slight reduction in $CD44^+CD62L^-$ effector Tconvs in DKO mice (Fig. 1C). In the small intestine we observed a reduction in the frequency of Tregs in DKO mice, indicating potential tissue specific effects (Fig. 1D). However, there was no observed reduction in the frequency of Tregs in the lungs of DKO mice (Fig. 1E). To gauge the suppressive capacity of DKO Tregs, we performed an *in vitro* suppression assay. $CD4^+YFP^+$ Tregs, sorted from the pooled secondary lymphoid organs (SLOs) of WT and DKO, were co-cultured with cell-trace stained $Ly5.1^+$ naïve $CD4^+$ T-cells at different ratios for 72 hours. At all ratios of Tregs and Tconvs, there was no significant difference in the suppressive capacity of WT

and DKO Tregs (Fig. 2A & B). Overall, this indicates that the loss of miR-23 family does not alter the suppressive capacity of Tregs at steady state but does lead to slight homeostatic differences in peripheral tissues, such as the small intestine.

Chapter 1, in part, includes unpublished data from Cheng-Jang Wu. The thesis author is the primary author of this thesis.

Chapter 2: Loss of miR-23 Clusters in Tregs Leads to Impaired Tumor Growth and a Reduction in Intratumoral Treg Frequencies with no Enhancement of T-cell Mediated Antitumor Responses

Our analysis of WT and DKO Tregs at steady state demonstrated a tissue-specific reduction of Treg frequencies within the small intestine. We hypothesized that the reduction of Tregs within the small intestine could potentially be reflected in a tumor setting, leading to enhanced antitumor immunity. To explore this possibility, we utilized different transplantable tumor models. Inoculating WT and DKO mice with B16-F10, MC38, E0771, and Sarcoma 9609 cancer cell lines, we found that DKO mice had reduced tumor kinetics for all models (Fig. 3). Further, we measured the expression levels of individual members of the miR-23 family in tumor and splenic derived Tregs from Foxp3-Thy1.1 reporter mice and found higher expression of each miR-23 family member in tumor derived Tregs compared to splenic Tregs (Fig. 4).

Examination of intratumoral immune cells in WT and DKO mice inoculated with Sarcoma 9609, we found that there was a reduction in the frequency of Tregs in DKO mice at day 14 (Fig. 5A & B). Examination of Foxp3 expression showed that there was no significant difference between WT and DKO intratumoral Tregs (Fig. 5B). These DKO intratumoral Tregs also had a modest reduction in the frequency of Treg-associated markers CD25, CD69, and CTLA-4 (Fig. 5E). Interestingly, we found that there was a reduction in the expression of proliferation associated peptide Ki67 in DKO intratumoral Tregs (Fig. 5C & D). The reduction in proliferative capacity of DKO Tregs potentially explains the reduction in intratumoral Tregs, though this does not exclude a role for impaired migration or viability within the tumor.

Next, we sought to determine the effector and antitumor responses of $CD8^+$ and conventional $CD4^+$ T-cells in the tumors of WT and DKO mice. We found that there was no significant difference in the frequency of $CD8^+$ and $CD4^+$ T-cells within the tumor (Fig. 6A). There

was also no alteration in the expression of Ki67 in $CD8^+$ and conventional $CD4^+$ T-cells (Fig. 6A). Surprisingly, when we examined the cytokine profile of $CD8^+$ and conventional $CD4^+$ T-cells at early time point (day 14), we found that there was no significant difference in the expression of IFNγ and TFNα, indicating no enhancement of antitumor immunity in DKO mice (Fig. 6B). To explain this contradictory result, we hypothesized that miR-23 family deficient Tregs are potentially more suppressive than their WT counterparts, which could compensate for the reduction in Treg frequency.

To determine if there was a difference in the suppressive capacity of intratumoral WT and DKO Tregs, we sorted intratumoral Tregs at day 21 and co-cultured them with cell-trace stained naïve $CD4^+$ T-cells at different Treg to Tconv ratios for 72 hours. Indeed, the suppressive capacity was increased in DKO intratumoral Tregs, with nearly a two-fold increase in suppressive capacity at a 1:1 ratio (Fig 7A & B). Considering our previous studies on ectopic overexpression of miR-27, which leads to reduced suppressive capacity by targeting the mRNA of IL-10 and Granzyme-B, we hypothesized that the increase in suppressive capacity was potentially due to derepression of *Il10* and *Gzmb* in DKO Tregs (Cruz et al., 2017). We found that there was an increase in the expression of *Il10* in DKO intratumoral Tregs, while *Gzmb* showed a non-significant trend of increase (Fig. 7C). FACS analysis of intratumoral DKO Tregs showed elevated expression of IL-10 and Gzmb (Fig. 7D). This supports the hypothesis that miR-23 family deficient intratumoral Tregs are more suppressive than their WT counterparts, which compensates for the reduction in DKO Treg frequency in the tumor, leading to similar $CD8^+$ and Tconv effector responses.

Chapter 2, in part, includes unpublished data from Cheng-Jang Wu. The thesis author is the primary author of this thesis.

Chapter 3: Natural Killer Cell Mediated Enhancement of Anti-Tumor Immunity in mice Harboring miR-23 Family Deficient Tregs

Natural Killers (NK) cells are a population of innate immune cells that mediate the apoptosis of target cells in an antigen-independent manner, making them key players in antitumor immunity. Previous studies on NK cells have demonstrated that IL-10, which normally serves as an immunosuppressive cytokine, induces the activation of NK cells, leading to an increase in IFNγ expression and lytic capabilities (Cai et al., 1999; Wang et al., 2021). Considering that miR-23 family deficient intratumoral Tregs have increased production of IL-10, we reasoned that NK cells could potentially show an increase in frequency and effector functions within the tumors of DKO mice.

Tumors from WT and DKO mice were taken down at day 21 for phenotyping of NK cells. Indeed, we found an increase in the frequency of $CD3\varepsilon^{-}NK1.1^{+}$ cells in the tumors of DKO mice (Fig. 8). A previous study examining the effect of IL-10 on NK cells in an MCMV infection model found that IL-10 prevented activation-induced cell death (AICD), leading to an increase in NK cell frequency (Stacey et al., 2011). Potentially, the increase in IL-10 in miR-23 family deficient intratumoral Tregs could enhance the survival of NK cells by preventing AICD, leading to improved antitumor responses.

To further determine if NK cells are playing a key role in the antitumor responses observed in DKO mice, we carried out an NK cell depletion study. In these experiments, tumor bearing WT and DKO mice were treated with NK1.1 depleting antibodies (250 µg doses) on days 0, 3, 7, 10, 14, and 17. Due to a limitation in the number of mice available, we were unable to set up isotype controls for these preliminary studies. In this setup, we see that the difference in tumor size observed between WT and DKO mice is reduced when NK cells are depleted (Fig 9A & B). We

confirmed by FACS analysis that NK ($CD3\varepsilon^{-}NKp46^{+}$) cells were depleted in the antibody treated mice (Fig. 9C). Taken together, the data suggests that NK cells play an important role in controlling the growth of tumors in DKO mice.

Chapter 4: Loss of miR-23 Clusters in Intratumoral Tregs Leads to an Increase in TCF1 and Alters Homeostasis

Due to the upregulation of previously identified targets of the miR-23 family in DKO intratumoral Tregs, we next investigated to see if other miR-23 family targets were upregulated. Surprisingly, we found that *Tcf7* expression was increased in miR-23 family deficient intratumoral Tregs (Fig. 10A). Our previous studies found that miR-24 targets *Tcf7* to control effector T-cell responses. This suggests that the loss of miR-23 family, specifically miR-24, leads to the derepression of *Tcf7* in intratumoral Tregs. Furthermore, we found that TCF1 protein was increased in DKO $TCF1^+$ intratumoral Tregs in comparison to their WT counterparts (Fig. 10B & 10D). Previous studies have found that TCF1 is downregulated in intratumoral Tregs and that loss of TCF1 in Tregs leads to increased tumor growth in a mouse model of colorectal cancer (Kidani et al., 2024; Osman et al., 2021). Gating on Tregs, we found that there was an increase in $TCF1^+$ cells in DKO mice (Fig.10C & 10D). When we gate on $CD4^+$ T-cells, we find that there is no difference in the frequency of $Foxp3^+TCF1^+$ Tregs in WT and DKO mice. Surprisingly, there was a reduction in $Foxp3^+TCF1^-$ Treg population and this reduction accounted for the loss of Tregs in DKO mice (Fig. 10E & 10F). This indicates that the increase in TCF1 protein, due to the derepression of *Tcf7* mRNA, leads to a loss in $TCF1^-$ intratumoral Tregs in DKO mice.

To study the role of TCF1 in intratumoral Tregs, we tested an adoptive transfer tumor model (Loo et al., 2020). We sorted $CD4^+YFP^+$ Tregs from the pooled SLOs of WT or DKO mice and transferred them into RAG-KO mice in combination with $CD4^+Foxp3^-$ and $CD8^+$ T-cells at a 1:1:1 ratio. The next day (24 hours) we inoculated these mice with Sarcoma 9609 and took down the tumors on day 21 for immunophenotyping. FACS analysis showed a reduction in intratumoral Tregs in RAG-KO mice that received DKO Tregs (Fig 11A & 11B). When quantifying the

frequency of Tregs out of live cells, we do see a significant reduction in the frequency of Tregs in the RAG-KO mice that received the DKO Treg mixture (Fig 11B). This indicates that the adoptive transfer tumor model can recapture the observed phenotype in our tumor-inoculated WT and DKO mice. This model provides us a means to overexpress TCF1, and other genes of interest, in wild-type Tregs or use shRNA knockdown in DKO Tregs to see if this phenocopies or ablates our observed phenotype, respectively.

Chapter 5: RNA-sequencing of Intratumoral miR-23 Family Deficient Tregs Reveals Reduced IL2-STAT5 and TGF-β Signaling Gene Signatures

To elucidate the molecular mechanisms that lead to altered Treg functions and homeostasis in tumors, we sorted WT and DKO Tregs from the tumors of female Foxp3-YFP-Cre heterozygous mice. These mice were inoculated with Sarcoma 9609 and Tregs were sorted at day 21, with a purity of >95%. Output Fastq files were aligned to the mm9 reference genome using the splice-aware aligner STAR (Dobin et al., 2013) and differential gene expression was analyzed using Deseq2 (Love et al., 2014).

Differential gene expression analysis found that a variety of genes were downregulated and upregulated in the miR-23 family deficient Tregs (Fig. 12A). Upon performing an unbiased Gene Set Enrichment Analysis (GSEA) on differentially expressed genes (DEGs) found a reduction in the Hallmark IL2-STAT5 and the TGF-β signaling pathway (Fig. 12B & Fig. 13A). Examination of the DEGs that could potentially explain the reduction in the IL2-STAT5 signaling pathway, we found that *Ecm1* was upregulated in miR-23 deficient Tregs (Fig. 12C). We confirmed *Ecm1* upregulation in intratumoral Tregs from DKO mice isolated on day 21 (Fig. 12D). Previous studies found that ECM1 binds to the IL2-Rβ subunit on T-cells to reduce IL2-STAT5 signaling, which leads to the enhancement of TFH cell differentiation (He et al., 2018). It has been well-recognized that Tregs express high levels of IL-2Rα (CD25) to deplete IL-2 in the tumor microenvironment promoting their own proliferation and suppressing effector T-cell responses. Re-examination of our day 14 tumor FACS data, we found that there was a preferential loss in $Foxp3^{+}CD25^{+}$ Tregs in comparison to $Foxp3^{+}CD25^{-}$ Tregs in the tumors of DKO mice. We hypothesized that $Foxp3^{+}CD25^{+}$ Tregs could have a reduction in proliferative capacity compared to $Foxp3^{+}CD25^{-}$ Tregs. Indeed, we observed reduced Ki67 expression in $CD25^{+}$ Tregs in DKO mice, while $CD25^{-}$

Tregs showed no alteration in proliferation between WT and DKO (Fig. 12E & 12F). This indicates that miR-23 family deficient intratumoral Tregs have reduced proliferative capacity, specifically within $CD25^+$ Tregs, which can be attributed to the ECM1-mediated impairment of the IL2-STAT5 signaling.

Next, we examined the DEGs that could lead to a reduction in TGF-β signaling in miR-23 family deficient intratumoral Tregs. In this analysis, we found that there was a reduction in expression of the integrin *Itgb8* (Fig. 13B). We confirmed *Itgb8* upregulation in intratumoral Tregs from DKO mice isolated on day 21 (Fig. 13C). Itgb8 is an integrin that associates with Itgav to form a heterodimer that functions to convert latent TGF-β into bioactive TGF-β (McCarty, 2020). Previous studies have found that Itgb8 is highly expressed on Tregs and plays a crucial role in regulating intestinal inflammation and in converting extracellular matrix-associated latent TGF-β to bioactive TGF-β in cancer (Dodagatta-Marri et al., 2021; Laine et al., 2021; Worthington et al., 2015). Furthermore, ECM1 has been demonstrated to interfere with heterodimers such as Itgav-Itgb8 in converting latent TGF-β to active TGF-β (Su et al., 2016). Overall, this supports the idea that miR-23 family deficient intratumoral Tregs have a reduced capacity to convert latent TGF-β to its active form. This could also explain the increase of NK cells in the tumors of DKO mice, as TGF-β has been demonstrated to be a powerful suppressor of NK cell responses (Viel et al., 2016).

DISCUSSION

Regulatory T-cells play an important role in impeding antitumor immune responses by conferring an immunosuppressive microenvironment within tumors. Uncovering the molecular mechanisms that regulate the homeostasis and functions of intratumoral Tregs is crucial in developing novel therapeutics to improve clinical outcomes. In this work, we utilized a transplantable model of Sarcoma to elucidate the functions of the miR-23 family in regulating intratumoral Tregs. We demonstrate that the loss of the miR-23 family leads to reduced tumor growth and alters the functions and homeostasis of intratumoral Tregs, supporting an NK cell-mediated antitumor response.

Previous studies in our laboratory demonstrated a crucial role for the miR-23 family in regulating the differentiation and function of T-cells. In this work, we found that loss of the miR-23 family in Tregs led to impaired tumor growth and a reduction in the frequency of Tregs within the tumor. Surprisingly, we observed no enhancement of antitumor $CD8^+$ and conventional $CD4^+$ T-cell responses. Furthermore, in vitro suppression assays found that miR-23 family deficient intratumoral Tregs were more suppressive than their WT counterparts, in part due to an increase in the expression of IL-10. This result is not unexpected given that our previous work found that overexpression of miR-27 in Tregs leads to reduced suppressive capacity, due to targeting of *Il10* and *Gzmb* (Cruz et al., 2017). Loss of miR-23 family in intratumoral Tregs ultimately leads to a derepression of *Il10*. This increase in suppressive capacity potentially explains the lack of T-cell mediated responses in the tumors of DKO mice, as it compensates for the reduction in intratumoral Treg frequency. Previous studies have demonstrated that IL-10 leads to enhanced NK cell responses and prevention of AICD in viral infection (Cai et al., 1999; Stacey et al., 2011; Wang et al., 2021). Analysis of NK cells in tumors found that were was an increase in NK cell frequency

in DKO mice; however, there was no apparent enhancement of IFNγ expression. This suggests that in the tumor setting, increased IL-10 leads to increased survival of NK cells by preventing AICD, enhancing antitumor immunity.

To further explore the role of IL-10 in regulating NK cells we have planned various *in vitro* and *in vivo* experiments to be carried out. In our *in vitro* system we plan to co-culture Tregs sorted from the tumors of WT and DKO mice with splenic NK cells, with or without IL-10 depleting antibodies. We hypothesize that the addition of IL-10-depleting antibodies would lead to a reduction in the IFNγ expression of NK cells co-cultured with DKO Tregs, as compared to controls. This system could demonstrate a direct role for DKO Treg-derived IL-10 in enhancing NK cell responses. To investigate the role of IL-10 *in vivo*, we will utilize the adoptive transfer tumor model. In this model, we would overexpress IL-10 in wild-type Foxp3-Thy1.1 reporter Tregs and adoptively transfer them, along with $CD4^+$ Tconvs and $CD8^+$ T-cells, into RAG-KO mice. The next day (24 hours) we would inoculate the mice with Sarcoma 9609 and profile the immune cell landscape on day 21. In this model, we would expect a reduction in T-cell antitumor immunity but an increase in NK cell frequencies. Furthermore, we can also use shRNA-mediated knockdown of IL-10 in DKO Tregs. In this setup, we would expect to see reduced NK cell-mediated responses but an increase in T-cell-mediated responses. Both *in vitro* and *in vivo* systems will provide critical evidence as to the role of IL-10 in NK cell-mediated antitumor immunity.

To further explore how the loss of miR-23 family in intratumoral Tregs leads to altered Treg homeostasis, we performed RNA-sequencing on Tregs sorted from heterozygous mice. In our analysis, we found a reduction in IL2-STAT5 and TGF-β signaling. In our RNA-seq results we identified upregulation of *Ecm1*, which mediates impairment of the IL2-STAT5 signaling by targeting IL-2Rβ (He et al., 2018). Impairment of IL2-STAT5 signaling was further corroborated

by a preferential loss of Foxp3^{+}CD25^{+} Tregs due to impaired proliferation. To account for the reduction in TGF-β signaling, we identified a reduction of *Itgb8*, an integrin that is highly expressed on Tregs and forms a heterodimer with Itgav to convert latent-TGF-β into its active form. ECM1 could also inhibit TGF-beta signaling by interfering with the Itgav-Itgb8 homodimers (Su et al., 2016).

To examine the role of reduced IL2-STAT5 in DKO Tregs, we plan on using *in vitro* and *in vivo* models. By sorting intratumoral and splenic Tregs from WT and DKO, we plan to incubate these Tregs with differing concentrations of IL-2 and stain pSTAT5 for FACS analysis. If DKO Tregs have reduced proliferation due to impaired IL2-STAT5 signaling, then we would expect reduced pSTAT5 levels at all concentrations of IL2, in comparison to WT Tregs. Using our adoptive transfer tumor model, we also plan on overexpressing constitutively active STAT5 to see if it rescues the impaired proliferative capacity of DKO Tregs, specifically in CD25^{+} Tregs. In this model, we would also expect to see an increase in the frequency of intratumoral Tregs due to rescuing the impaired proliferation of DKO Tregs. To test the role of reduced TFG-β signaling in Tregs, we plan to utilize a bioactive TGF-β reporter cell line. Culturing intratumoral Tregs from WT and DKO with latent-TGF-β, we would transfer the supernatant to the TGF-β reporter cell line culture and measure the relative bio-activity of TGF-β. Due to the reduction in *Itgb8* and increase in *Ecm1*, we expect DKO Tregs to have a reduced capacity to convert latent-TGF-β into its bioactive form. These *in vitro* and *in vivo* assays can further validate the importance of IL2-STAT5 and TGF-β signaling in regulating the homeostasis and functions of DKO intratumoral Tregs.

Furthermore, our RNA-seq results also found that *Tcf7* was increased. In effector T-cells, we had previously demonstrated that miR-24 targets *Tcf7* (Cho et al., 2017). We confirmed that

TCF1 expression was increased both at the mRNA and protein level, leading to an increase in $TCF1^+$ Tregs and a loss of $TCF1^-$ Tregs. To determine the functional role of TCF1 in DKO Tregs, we plan to utilize the adoptive transfer tumor model. In one setup, we plan to overexpress the long-form of TCF1 (L-TCF1) in wild-type Tregs isolated from Foxp3-Thy1.1 reporter mice and adoptively transfer, in combination with $CD4^+$ Tconvs and $CD8^+$ T-cells, into RAG-KO mice. The next day (24 hours), we would inoculate them with Sarcoma 9609 and analyze the immune cell landscape on days 14 and 21. In this model, if TCF1 plays a role in reducing intratumoral Treg frequency, we would expect an increase in $TCF1^+$ Tregs but a loss in $TCF1^-$ Tregs, leading to enhanced antitumor immunity of $CD8^+$ T-cells and endogenous NK cells. In another model, we plan on using shRNA-mediated knockdown of TCF1 in DKO Tregs. In this setup, we would expect an increase in intratumoral Tregs, with a further reduction in T-cell mediated responses and potentially further increases in NK cell frequencies. This is in part due to the increase of *Il10* resulting from the loss of miR-27, which we predict would be independent of TCF1 increase. To determine if the miR-23 family indirectly regulates the DEGs observed in our RNA-seq analysis by targeting TCF1, we plan to sort intratumoral Tregs from both models and measure gene expression using RT-qPCR. Using this *in vivo* system, we can further dissect the molecular mechanisms underlying miR-23 family in regulating intratumoral Tregs.

In this study, we characterized the homeostatic and functional consequences of miR-23 family ablation in intratumoral Tregs. Our current evidence suggests that the miR-23 family potentially functions as a rheostat by controlling both Treg homeostasis through targeting TCF1 and by modulating suppressive capacity by targeting IL-10. Further studies must be conducted to validate this functional role of the miR-23 family. The proposed *in vitro* and *in vivo* models will further elucidate the direct and indirect mechanisms that the miR-23 family plays in controlling

intratumoral Treg responses. By uncovering these mechanisms, our research could uncover crucial miR-23 family regulatory pathways in intratumoral Tregs and provide new avenues for therapeutic development.

MATERIALS & METHODS

Mice

miR-23Ca/b$^{fl/fl}$ mice were crossed to *Foxp3-YFP-Cre* mice to obtain Treg-specific deletion (Treg-DKO or DKO) of both miR-23a and miR-23b clusters. To generate heterozygous mice, Treg-DKO female mice were crossed with male *miR-23Ca/b*$^{fl/fl}$ mice. All mice were maintained and handled in accordance with the Institutional Animal Care and Use Guidelines of University of California San Diego and National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and the Animal Research: Reporting In Vivo Experiments (ARRIVE) guidelines.

Cell Lines & Tumor Inoculation

Sarcoma 9609 cell lines, generously provided by Bui Lab, was passaged and frozen in FBS supplemented with DMSO (10%). Cell were cultured in RPMI (500 mL) supplemented with 5 mL of penicillin/streptomycin, 5 mL of L-glutamine, 0.5 mL of β-ME, 5 mL of NEAA, 5 mL of Sodium Pyruvate, 10 mL of HEPES, and 50 mL of FBS. Frozen cell lines were unthawed and passaged three times before inoculation into mice. Cells were counted on a hemocytometer and diluted to a concentration of 10 million cells per milliliter. Mice were subcutaneously inoculated in their flanks with 100 microliters of resuspended tumor cell solution. Tumors were measured with digital calipers when tumors were palpable and measured every two days. Mice were sacrificed on day 14 for early time point analysis and on day 21 for late time point analysis.

Tissue Preparation

Single-cell suspensions of spleens and lymph nodes were prepared by mechanical disruption using frosted slides in PBS. Single cells were filtered through a 70-micron mesh and resuspended to working volumes. Intratumoral immune cells were isolated by dicing tumor

samples into fine pieces and were incubated in RPMI supplemented with Liberase LT (0.1 mg/mL), DNase-I (0.05%), and HEPES (20 mM) for 30 minutes at 37 degrees Celsius at 190 rpm. Digested tissues were inactivated by ice-cold RPMI supplemented with HEPES (20 mM) and BSA (0.5%). After inactivation, digested tissues were filtered through 100-micron filters using a 1-mL syringe plunger. Isolated samples were spun for 10 minutes at 1500 rpm at 4 degrees Celsius. Pelleted cells were resuspended in 47% Percoll and spun at 1500 rpm at 4 degrees Celsius for 10 minutes to isolate purified immune cells. Purified samples were then resuspended in EDTA-supplemented RPMI and used for downstream staining for flow cytometric analysis and/or cell sorting.

Flow Cytometry and Antibodies

For all samples, cells were stained with Ghost Dye Red 780 (Tonbo Biosciences) for 15 to 20 minutes at 4 Celsius in the dark. Surface staining buffer consisted of 3 part FACS buffer and 1 part 2.4G2. After incubation cells were washed with FACS buffer. The following antibodies were used for surface staining CD4-PerCP-Cy5.5, CD8α-BV785, CD44-eFluor 450, CD62L-BV711, CD25-PE, CD69-PE-Cy7, CD3e-BV785, NKp46-APC, NK1.1-APC, Thy1.1-PE, and CD45-BV510. Cells were stained for 15 minutes at 4 degrees Celsius in that dark before being washed with FACS buffer. FOXP3/Transcription Factor Staining Kit was used for intracellular staining following the manufacturer's instructions (Tonbo Biosciences). After fix/perm cells were washed with 10X Perm Buffer diluted with water. The intracellular staining buffer consisted of 3 parts 1X Perm Buffer and 1 part 2.4G2. The following antibodies were used for intracellular staining: Foxp3-FITC, IL10-PE, Gzmb-APC, CTLA4-PE, TCF1-APC, Ki67-APC, IFNγ-PE-Cy7, IFNγ-APC, and TNFα-PE-Cy7. Samples were analyzed on a BF-Fortessa and BD Fortessa X-20 platforms. Cell sorting was performed on BD FACSAria-III platform.

Quantitative Real-Time qPCR

For detecting the expression of different genes, FACS-sorted regulatory T-cells ($CD4^+YDP^+$ or $CD4^+Thy1.1^+$) and conventional cells ($CD4^+YFP^-$ or $CD4^+Thy1.1^-$) were frozen in Trizol (Thermo Fischer Scientific). Total RNA was extracted using chloroform and isopropanol-based separation. Complementary DNA (cDNA) was generated using the iScript cDNA synthesis kit (Bio-Rad Laboratories). MicroRNA cDNA was generated using a TaqMan microRNA cDNA synthesis kit (Applied Biosystems) for the detection of the miR-23 family members. Real-time qPCR was performed on iScript-generated cDNA using SYBR Green PCR kits (Applied Biosystems). Real-time qPCR was performed on Taqman-generated microRNA cDNA using TaqMan Universal PCR Master Mix (Applied Biosystems). All real-time reactions were run on a 7900HT Fast Real-Time PCR system (Thermo Fischer Scientific). The primer sequences used are as follows: GAPDH: 5'-CGTCCCGTAGACAAAATGGT-3' (F) & 5'-TCAATGAAGGGGTCGTTGAT-3' (R), FOXP3: 5'-GCGAAAGTGGCAGAGAGGTA-3' (F) & 5'-TCCAAGTCTCGTCTGAAGGC-5' (R), ECM1: 5′-GCCAGCTCTGTGGAAGTGGA-3′ (F) & 5′-CCGGAATCTGTTTATGCTTGC-3′ (R), ITGB8: 5′-ACTTCTCCTGTCCCTATCTCCA-3′ (F) & 5′-ATCTGCCACCTTCACACTCC-3′ (R) , TCF1: 5'-TCAATCTGCTCATGCCCTAC-3' (F) & 5'-TGGACTGCTGAAATGTTCGT-3' (R), IL10: 5'-CAGAGCCACATGCTCCTAGA-3' (f) & 5'-TGTCCAGCTGGTCCTTTGTT-3' (R), TGF-β1: 5'-GGAGAGCCCTGGATACCAAC-3' (F) & 5'-AAGTTGGCATGGTAGCCCTT-3' (R)

RNA-sequencing and Differential Gene Expression Analysis

Heterozygous WT (*Foxp3-Cre/+*) and DKO (*Foxp3-Cre/+ miR-23Ca/b*$^{fl/fl}$) mice were inoculated in left and right flanks with 1 million Sarcoma 9609 cells in 100 µL of PBS. Immune cells were isolated from the tumors and $CD4^+YFP^+$ Tregs were sorted at a purity of >95%. Samples

were frozen in Trizol and RNA was extracted for cDNA library preparation. Fastq files were quality-controlled using the Fastp (Chen et al., 2018) program and the resulting trimmed Fastq files were aligned to the mm9 mouse reference genome using the splice-aware aligner STAR (Dobin et al., 2013). Direct count outputs were organized into a text file and analyzed using DEseq2 (Love et al., 2014). Volcano plots were generated using the R-package EnhancedVolcano (Blighe et al., 2023) and GSEA analysis was performed using the R-package Fsgea (Korotkevich et al., 2019).

In Vitro Suppression Assay

$CD4^+YFP^+$ Regulatory T-cells were sorted from the pooled SLOs of WT and Treg-DKO mice. Tregs were sorted to a purity of >95%. Naïve conventional $CD4^+$ T-cells were enriched using a Naïve Mojosort Enrichment Kit (Miltenyi) and stained with Cell-Trace Dye for 10 minutes at 37 degrees Celsius. Cells were subsequently diluted in 10% cRPMI on ice for 5 minutes and washed 3 times with 10% cRPMI. Both Tregs and Tconvs were resuspended to a concentration of 1 million cells per mL. Antigen-presenting cells were isolated from the spleens of B6 mice using a CD90.2 Depletion Kit (Miltenyi). Isolated $CD90.2^-$ cells were treated with Mitomycin-C for 30 minutes at 37 degrees Celsius before being washed 3 times with 10% cRPMI. APCs were subsequently prepared at a concentration of 3 million cells per mL. Anti-CD3 stock was diluted in cRPMI to a concentration of 4µg/mL. Tconvs (50K per well) were co-cultured with Tregs at the indicated ratios in the presence of Mitomycin-C treated APCs (150K per well) in 10% cRPMI supplemented with anti-CD3 (1 µg/mL). Cells were co-cultured in a 96-well round bottom plate for 72 hours in an incubator at 37 degrees Celsius and 5% CO_2 before analysis.

Adoptive Transfer Tumor Model

CD4+YFP+ Regulatory T-cells were sorted from WT and Treg-DKO mice from pooled secondary lymphoid organs. $CD8^+$ T-cells and $CD4^+Thy1.1^-$ Tconvs were sorted from the pooled secondary lymphoid organs of Foxp3-Thy1.1 reporter mice. WT or DKO Tregs, $CD8^+$ T-cells, and $CD4^+Thy1.1^-$ Tconvs were mixed at a 1:1:1 ratio and adoptively transferred into RAG-KO mice through retro-orbital injection. 24 hours later the mice were inoculated in their right flanks with 1 million Sarcoma 9609 cells in 100 µL. Mice were taken down 21 days later for analysis of tumor immune cells.

Statistical Analysis

Unpaired Student's t-test was done on all reported data using Prism software (GraphPad). (*P<0.05; **P<0.01, ***P<0.0001, **** P<0.00001)

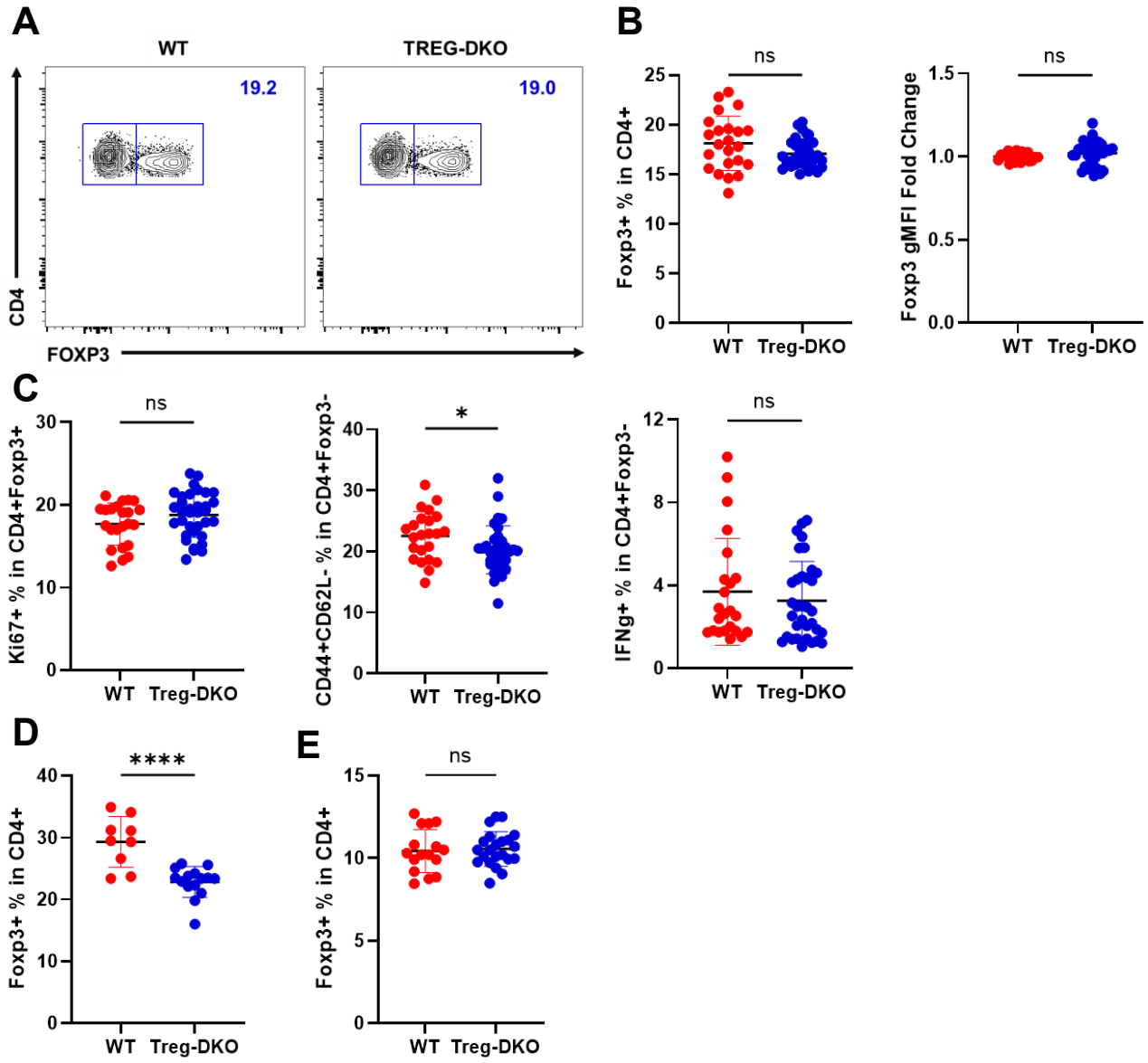

Figure 1. No Apparent Immune Phenotype in Mice with Treg-Specific Ablation of miR-23 Clusters at Steady State. (A) FACS analysis, (B) frequency of Foxp3$^+$ cells in CD4$^+$ T-cells and fold-change of Foxp3 gMFI compared to WT. (C) Frequency of Ki67$^+$ cells in CD4$^+$Foxp3$^+$ Tregs, CD44$^+$CD62L$^-$ effector Tconvs, and IFNγ$^+$ cells in CD4$^+$Foxp3$^-$ conventional T-cells in the spleen. Frequency of Foxp3$^+$ cells in CD4$^+$ cells in the (D) small intestine (SI) and (E) lung. Data represented as the mean ± S.D.

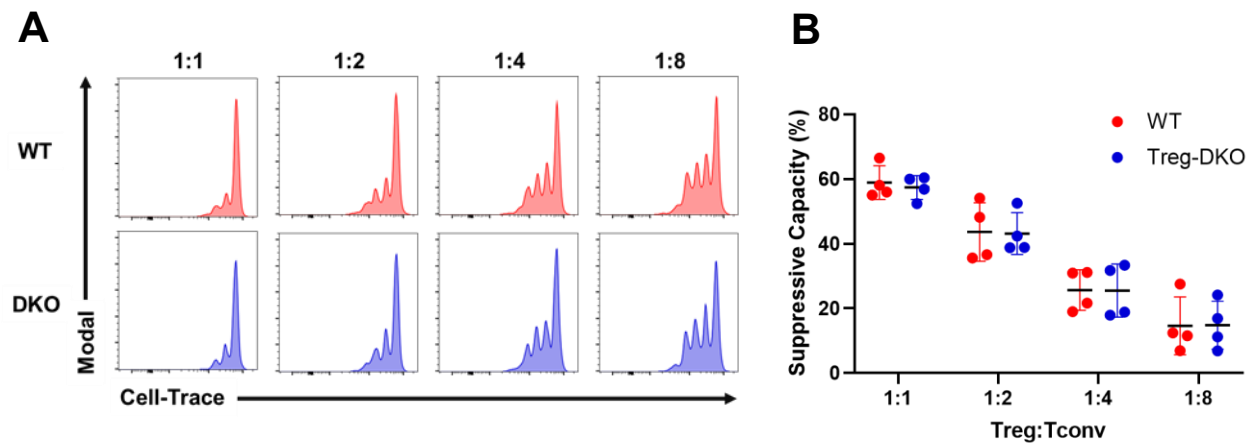

Figure 2. Comparable Suppressive Capacity in Treg Cells with or without miR-23 Clusters. (A) FACS analysis of cell-trace stained Tconvs co-cultured with Tregs at the indicated ratios after 72 hours of culture. (B) Quantification of the suppressive capacity of WT and DKO Tregs at the indicated Treg to Tconv ratios. The suppressive capacity was calculated using the following formula: Suppression =100 x [Average (Negative Control) – Average (Sample)] / [Average (Negative Control)]. Data represented as the mean ± S.D.

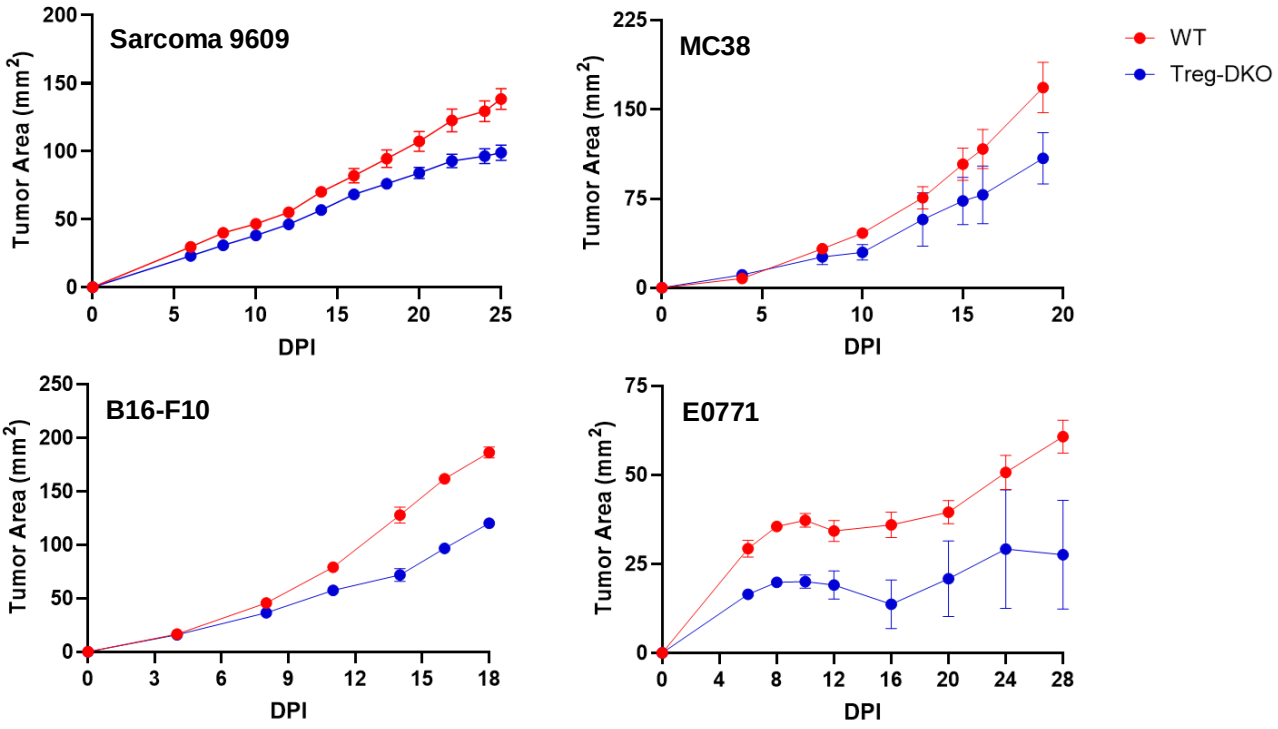

Figure 3. Reduced Tumor Growth in Mice with Treg-Specific Ablation of miR-23 Clusters. Tumor area kinetics, calculated as W × L, for WT and DKO mice inoculated with the cancer cell lines Sarcoma 9609, MC38, B16-F10, and E0771. 1 million cancer cells suspended in 100 µL of PBS were injected into the right flank of mice and were measured with digital calipers when palpable. Data represented as the mean ± SEM.

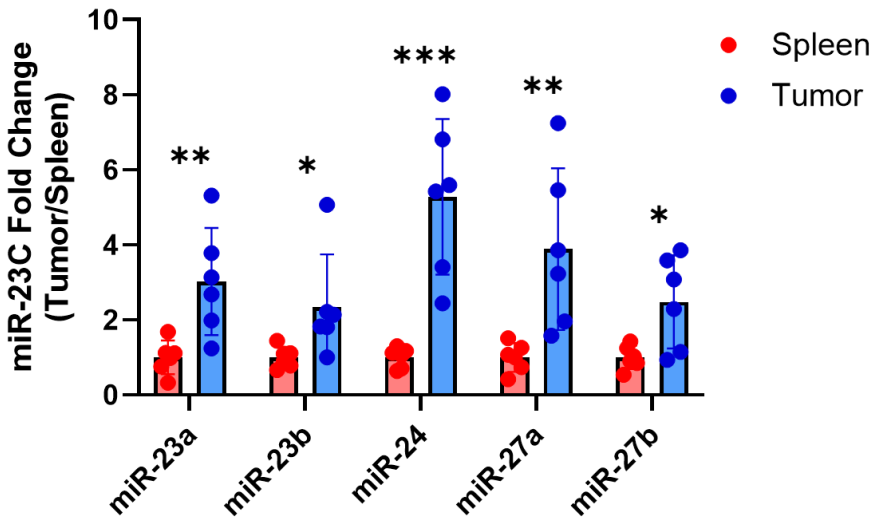

Figure 4. Further Elevation of miR-23 Cluster Expression in Tumor-Infiltrating Tregs in Comparison to Splenic Tregs. The expression of individual members of the miR-23 family was measured in Tregs sorted at day 14 from the tumor and spleen from Foxp3-Thy1.1 mice inoculated with Sarcoma 9609. Fold change was calculated as the ratio of tumor and splenic Treg miRNA relative expression. Data represented as the mean ± S.D.

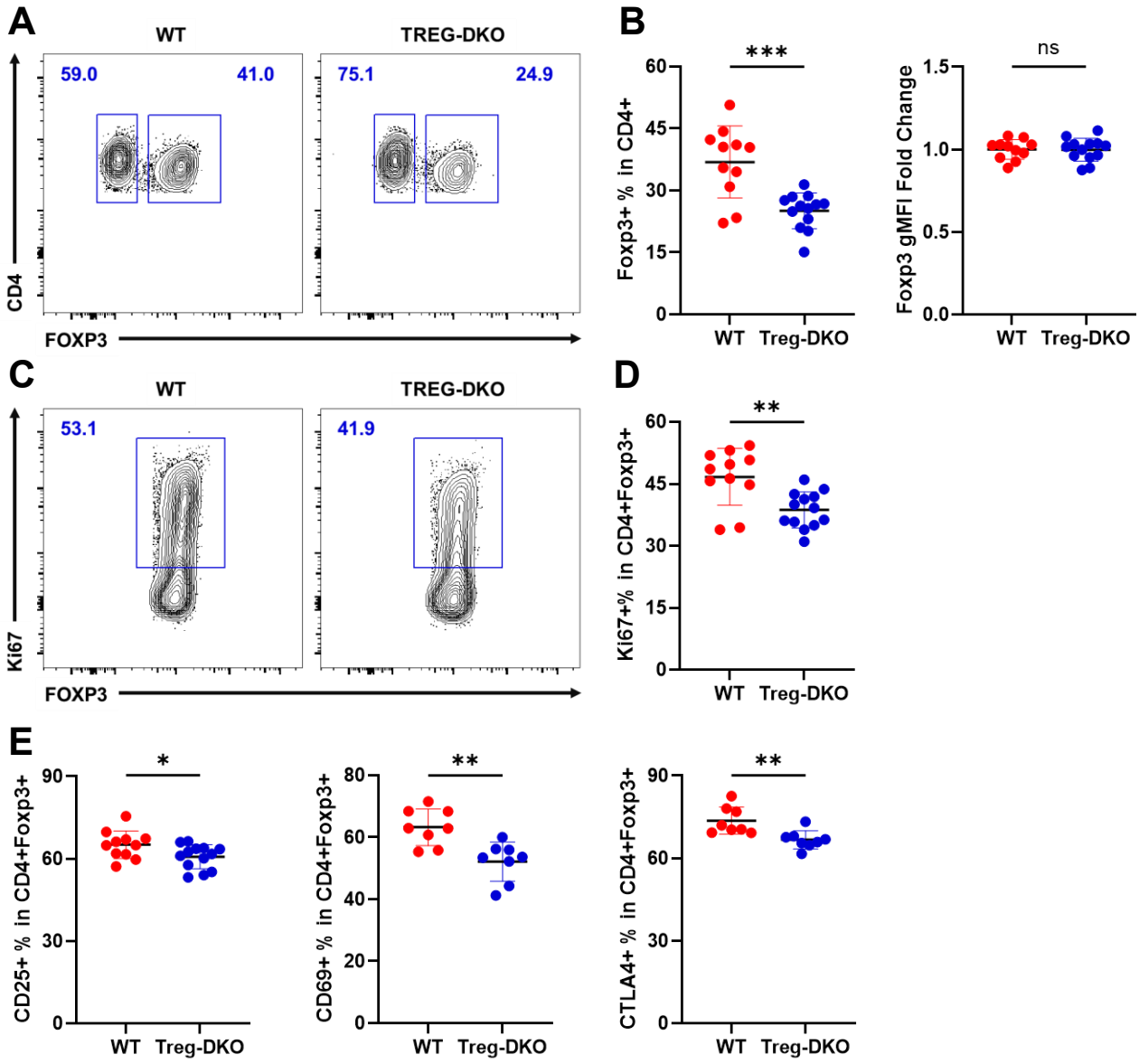

Figure 5. Ablation of miR-23 Clusters Leads to a Reduction in the Frequency of Tumor-Infiltrating Tregs. WT and DKO mice were inoculated with Sarcoma 9609 and tumor immune cells were isolated on day 14. (A) FACS analysis of Foxp3$^+$ cells in CD4$^+$ T-cells in WT and DKO tumors. (B) Treg frequency and fold-change of Foxp3 gMFI compared to WT. (C) FACS analysis of Ki67$^+$ cells in CD4$^+$Foxp3$^+$ Tregs. (D) Frequency of Ki67$^+$ cells in CD4$^+$Foxp3$^+$ Tregs. (E) Frequency of CD25$^+$, CD69$^+$, and CTLA-4$^+$ cells in CD4$^+$Foxp3$^+$ Tregs. Results are represented as mean ± S.D.

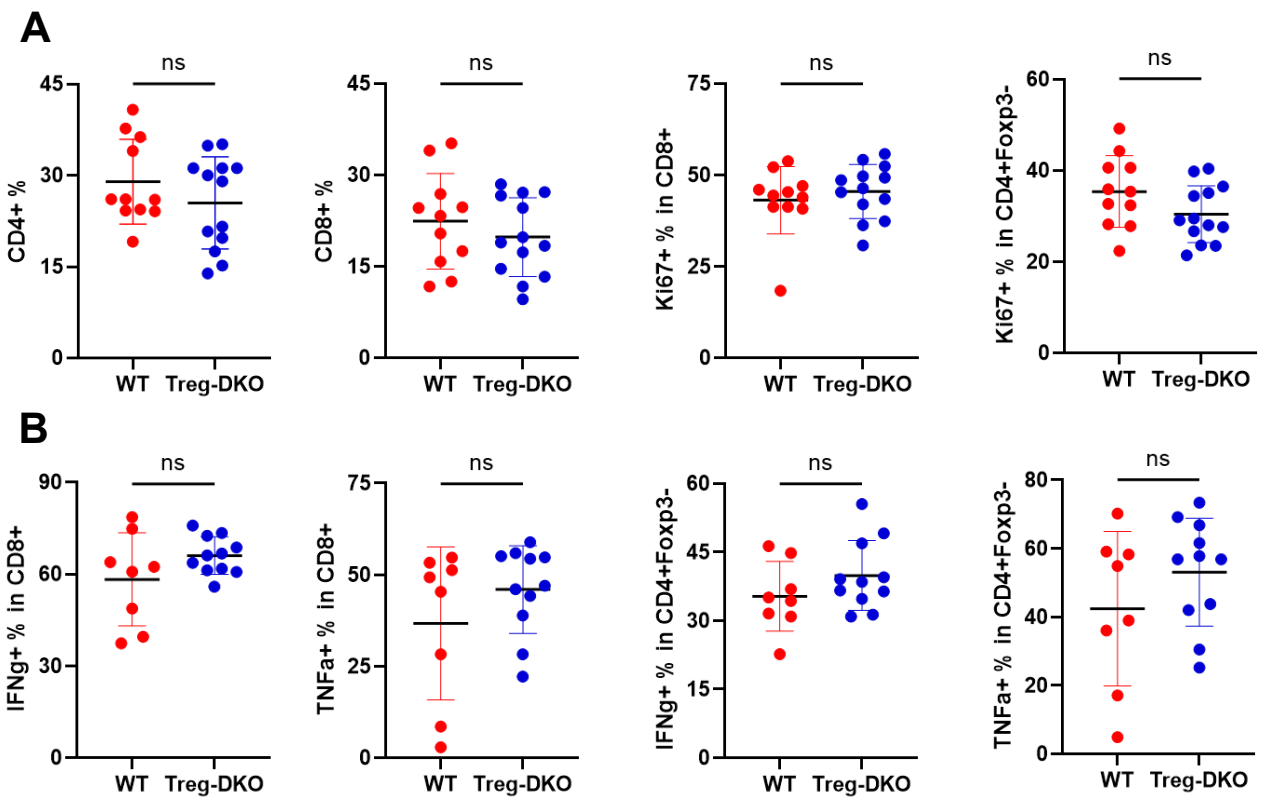

Figure 6. Minimal Difference in Effector T-cell Responses in the Tumor. Immune cells isolated from day 14 tumors from WT and DKO mice were analyzed by FACS to quantify effector T-cell and cytokine profiles. (A) Frequency of $CD4^+$ and $CD8^+$ T-cells, as well as the frequency of $Ki67^+$ cells in $CD4^+Foxp3^-$ Tconvs and $CD8^+$ T-cells. (B) The frequency of $IFN\gamma^+$ and $TNF\alpha^+$ cells in $CD4^+Foxp3^-$ Tconvs and $CD8^+$ T-cells. Results are represented as mean ± S.D.

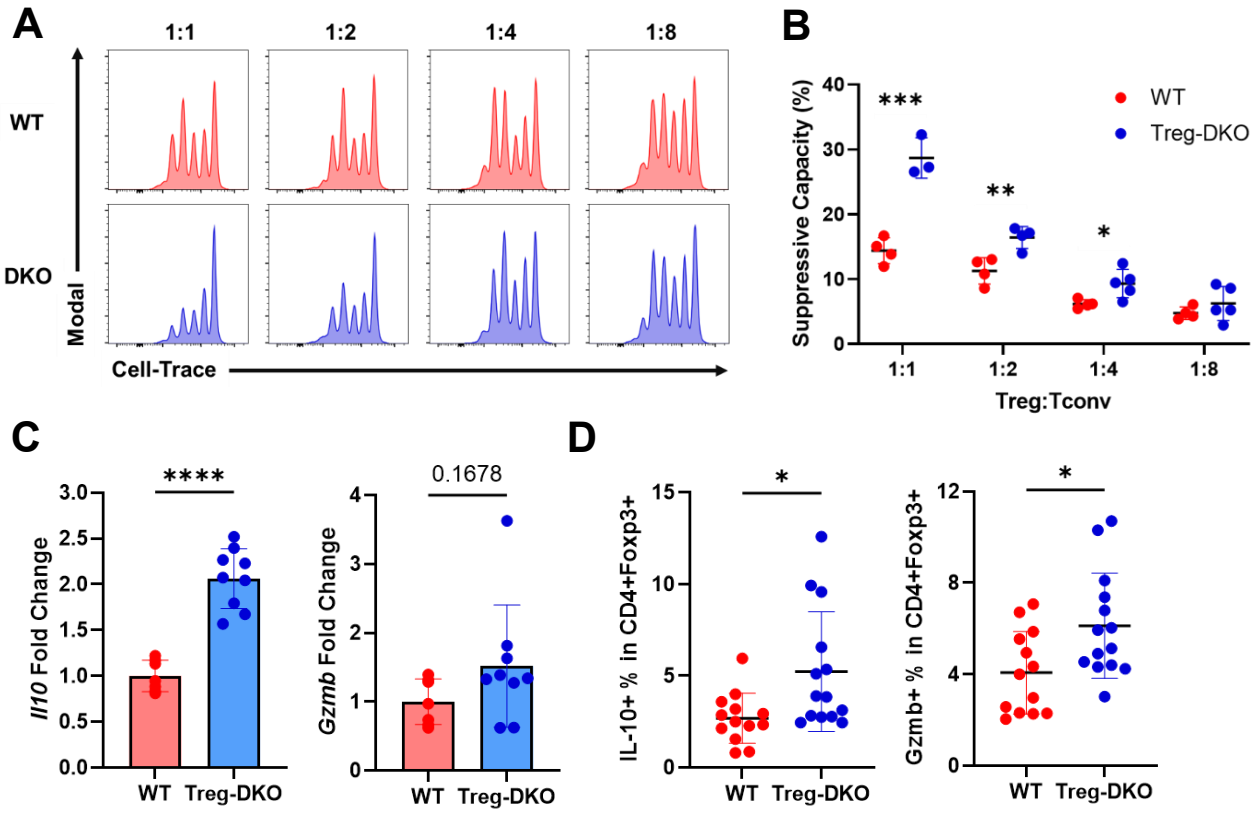

Figure 7. Enhanced Suppressive Capacity in miR-23 Cluster-Deficient Intratumoral Tregs. (A) FACS analysis of cell-trace stained Tconvs co-cultured with day 21 intratumoral Tregs at the indicated ratios after 72 hours of culture. (B) Quantification of the suppressive capacity of WT and DKO Tregs at the indicated Treg to Tconv ratios. (C) Fold-change of *Il10* and *Gzmb* gene expression in intratumoral Tregs of WT and DKO mice, as determined by RT-qPCR. (D) Frequency of IL-10^{+} and Gzmb^{+} cells in CD4^{+}Foxp3^{+} from the tumors of WT and DKO at day 21. Results are represented as mean ± S.D.

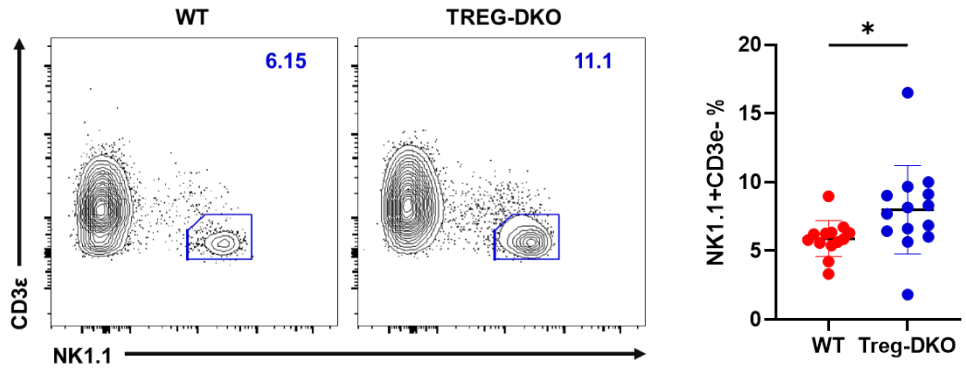

Figure 8. Elevated Intratumoral NK cell Frequencies were Found in Mice with Treg-Specific Deletion of miR-23 Clusters. WT and DKO mice were inoculated with Sarcoma 9609 and NK cell populations were examined at day 21. FACS analysis (left) and frequency (right) of $CD3\varepsilon^{-}$ $NK1.1^{+}$ cells in the day 21 tumors of WT and DKO. Results are represented as mean ± S.D.

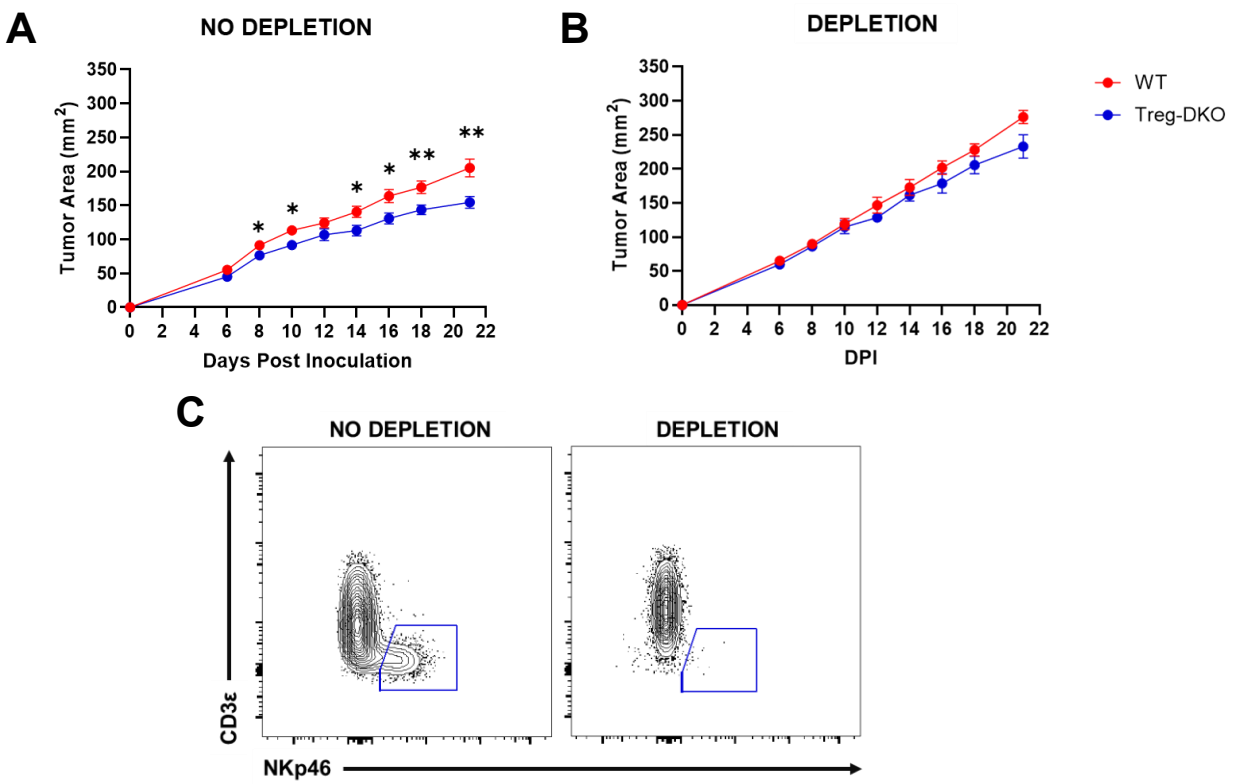

Figure 9. Depletion of NK cells Abolishes the Difference in Tumor Growth Between WT and DKO mice. WT and DKO mice were inoculated with Sarcoma and administered NK1.1 depleting antibodies (250 µg per mouse) on days 0, 3, 7, 10, 14, and 17. (A) Tumor growth in WT and DKO mice from day 21 immune cell profiling studies. (B) Tumor growth in WT and DKO mice that were depleted of NK cells. (C) FACS analysis of $CD3\varepsilon^{-}NKp46^{+}$ NK cells in non-depleted and depleted mice. Results are represented as mean ± SEM.

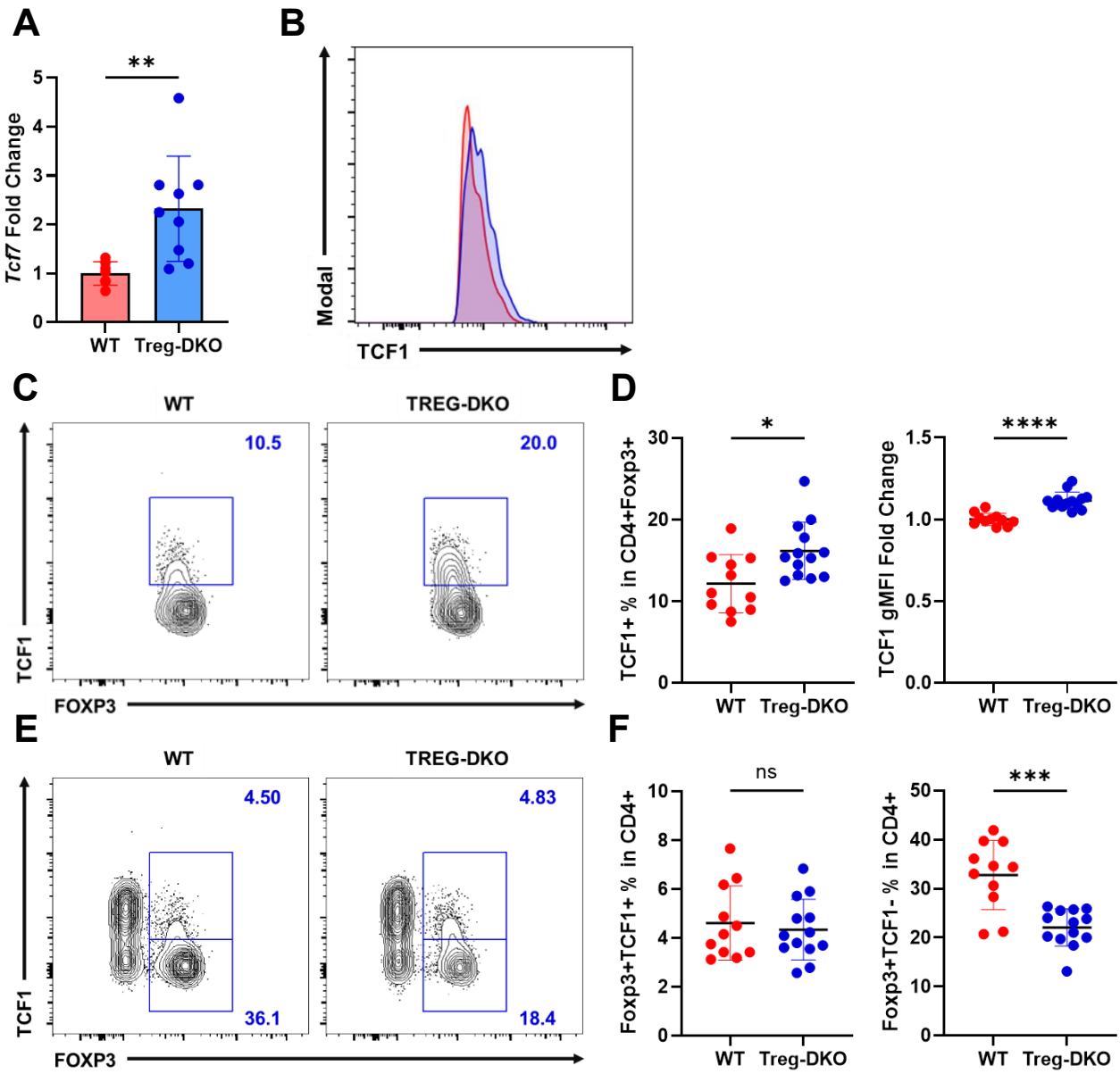

Figure 10. Ablation of miR-23 Clusters in Tregs Leads to a Derepression of TCF1 and a Selective Loss of TCF1$^-$ Effector-Like Tregs in the Tumor. (A) RT-qPCR of *Tcf7* expression in day 21 intratumoral Tregs from WT and DKO mice. (B) FACS histograms of TCF1 expression in WT (red) and DKO (blue) TCF1$^+$ Tregs from day 14 tumors. (C) FACS analysis of TCF1$^+$ cells in CD4$^+$Foxp3$^+$ cells from the day 14 tumors of WT and DKO mice. (D) Frequency of TCF1$^+$ cells in CD4$^+$Foxp3$^+$ Tregs and fold-change in the TCF1 gMFI of TCF1$^+$ Tregs. (E) FACS analysis of Foxp3$^+$TCF1$^+$ and Foxp3$^+$TCF1$^-$ Tregs, gated on CD4$^+$ T-cells. (F) Frequency of Foxp3$^+$TCF1$^+$ and Foxp3$^+$TCF1$^-$ Tregs. Results are represented as mean ± S.D.

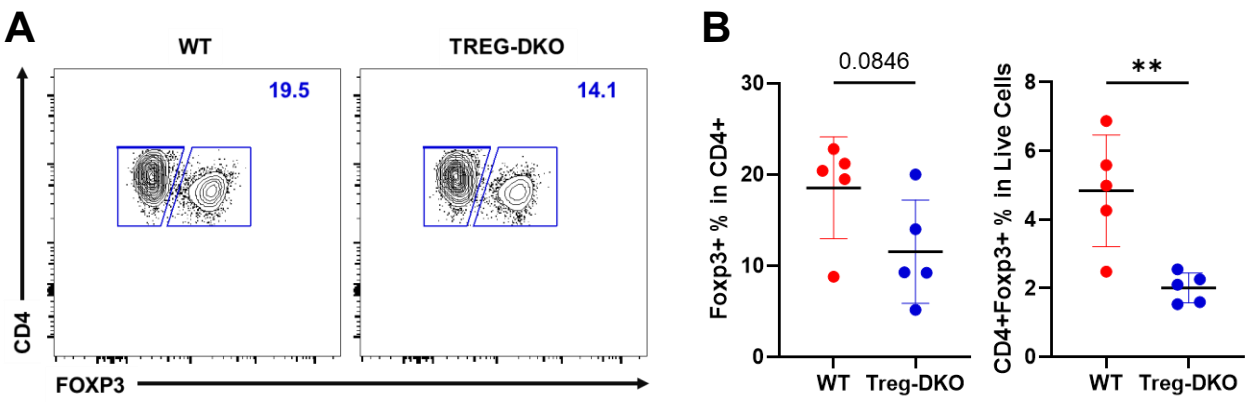

Figure 11. Reduced Infiltration of miR-23 Cluster Deficient Tregs Observed in Adoptive Transfer Tumor Model. RAG-KO mice received WT or DKO Tregs mixed with $CD4^{+}Thy1.1^{-}$ Tconvs and $CD8^{+}$ T-cells at a 1:1:1 ratio by retro-orbital injection 24 hours before tumor inoculation. (A) FACS analysis of $Foxp3^{+}$ cells in $CD4^{+}$ T-cells from the day 21 tumors in adoptively transferred RAG-KO mice. (B) Frequency of $Foxp3^{+}$ cells in $CD4^{+}$ T-cells and out of total live cells. Results are represented as mean ± S.D.

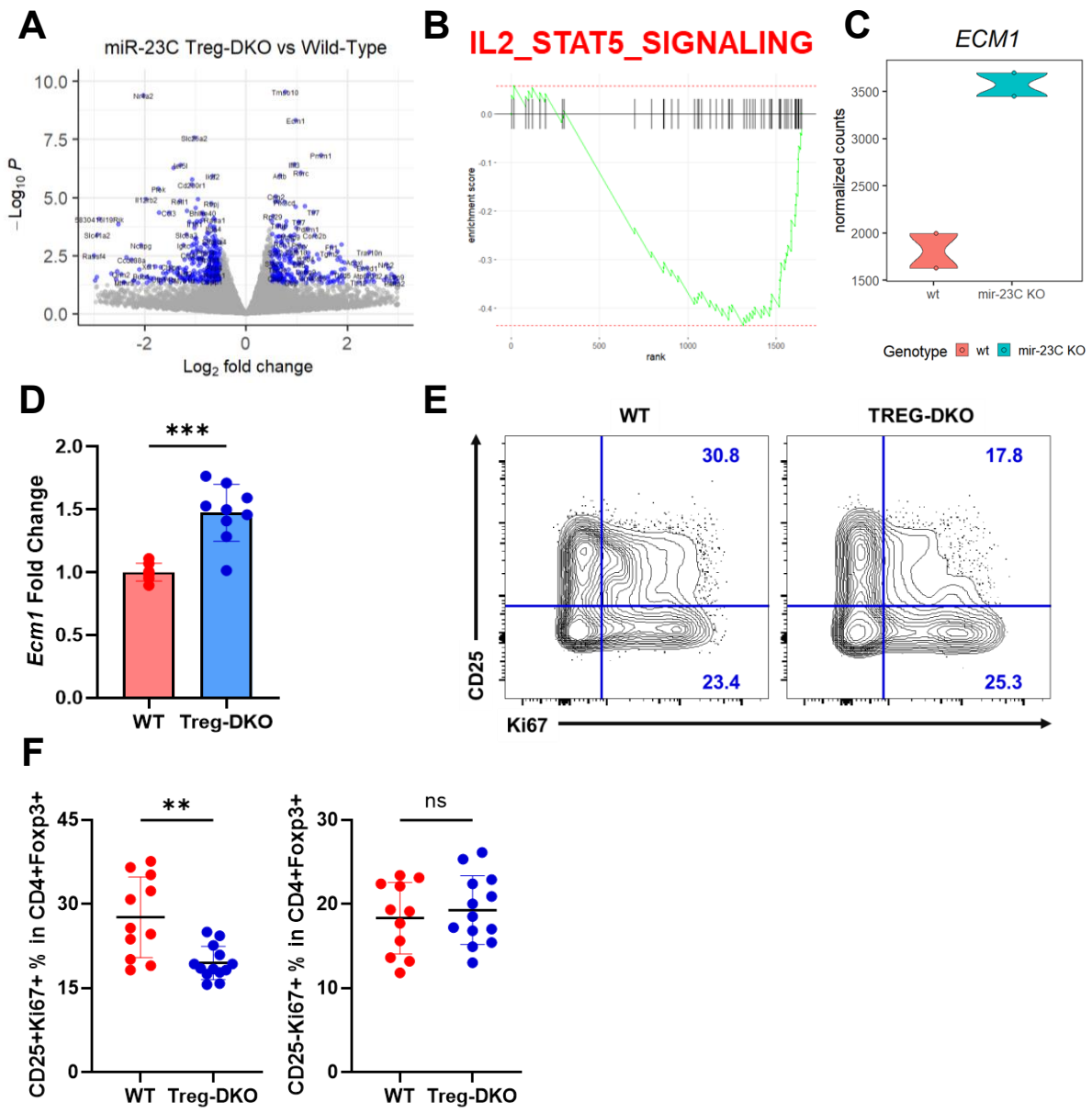

Figure 12. RNA-seq Analysis of Tumor-Infiltrating Tregs Points to the Role of miR-23 Clusters in Regulating IL2-STAT5 Signaling. (A) Volcano plot of differentially expressed genes between DKO and WT Tregs. (B) Gene set enrichment analysis for IL2-STAT5 signaling between DKO and WT Tregs. (C) Counts of *Ecm1* between DKO and WT Tregs from RNA-seq data. (D) Fold-change of *Ecm1* expression in day 21 intratumoral Tregs from WT and DKO mice, as determined by RT-qPCR. (E) FACS analysis of $CD25^{+}Ki67^{+}$ and $CD25^{-}Ki67^{+}$, gated on $CD4^{+}Foxp3^{+}$ Tregs, in the day 14 tumors of WT and DKO mice. (F) Frequency of $CD25^{+}Ki67^{+}$ and $CD25^{-}Ki67^{+}$ cells in $CD4^{+}Foxp3^{+}$ Tregs. Results are represented as mean ± S.D.

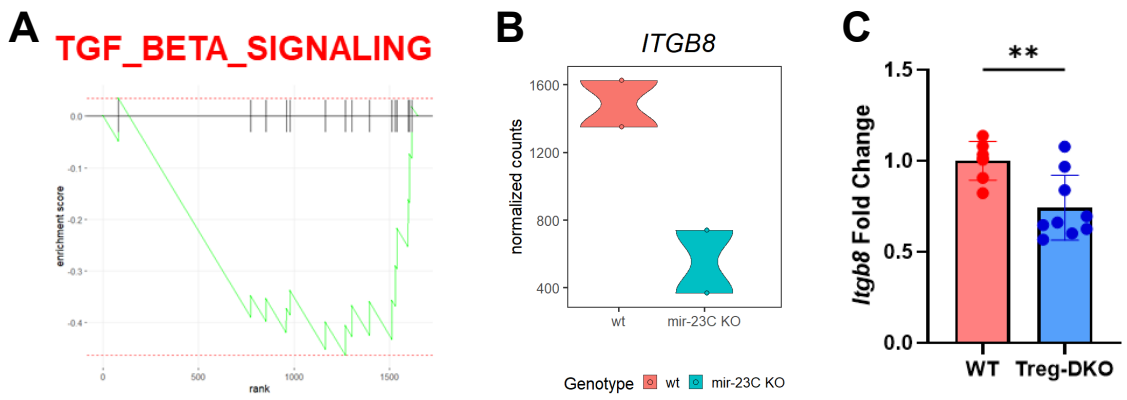

Figure 13. Loss of miR-23 Clusters in Intratumoral Tregs Leads to a Reduction in TGF-β Signaling. (A) Gene set enrichment analysis of TGF-β signaling. (B) Counts of *Itgb8* between DKO and WT Tregs from RNA-seq data. (C) Fold-change of *Itgb8* expression in day 21 intratumoral Tregs from WT and DKO mice, as determined by RT-qPCR. Results are represented as mean ± S.D.

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