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miRNAs regulate T cell production of IL-9 and identify hypoxia inducible factor-2 α (HIF-2 α) as an important regulator of Th9 and Treg differentiation

Short Title: Role of microRNAs in helper T cell development

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Abbreviations: miRNAs, microRNAs; IL-9 Interleukin-9; Th helper T cell; Treg, regulatory T cell; iTreg, in vitro–induced Treg; HIF, hypoxia inducible factor; qRT-PCR, quantitative RT-PCR; UTR untranslated region; Tcon, conventional helper T cell.

SUMMARY

MicroRNAs (miRNAs) regulate many aspects of helper T cell (Th) development and function. Here we found they are required for suppression of interleukin-9 (IL-9) expression in Th9 cells and other Th subsets. Two highly related miRNAs (miR-15b and miR-16) that we previously found to play an important role in regulatory T cell (Treg) differentiation were capable of suppressing IL-9 expression when they were overexpressed in Th9 cells. We used these miRNAs as tools to identify novel regulators of IL-9 expression and found they could regulate the expression of *Epas1*, which encodes hypoxia inducible factor (HIF)-2 α . HIF proteins regulate metabolic pathway usage that is important in determining appropriate Th differentiation. The related protein, HIF-1 α enhances Th17 differentiation and inhibits Treg differentiation. Here we found that HIF-2 α was required for IL-9 expression in Th9 cells, but its expression was not sufficient in other Th subsets. Furthermore, HIF-2 α suppressed Treg differentiation like HIF-1 α , demonstrating both similar and distinct roles of the HIF proteins in Th differentiation and adding a further dimension to their function. Ironically, even though miR-15b and miR-16 suppressed HIF-2 α expression in Tregs, inhibiting their function in Tregs did not lead to an increase in IL-9 expression. Therefore, the physiologically relevant miRNAs that regulate IL-9 expression in Tregs and other subsets remain unknown. Nevertheless, the analysis of miR-15b and miR-16 function led to the discovery of the importance of HIF-2 α so this work demonstrated the utility of studying miRNA function to identify novel regulatory pathways in helper T cell development.

INTRODUCTION

IL-9 is a cytokine that has recently received a significant amount of attention for its role in tumour immunity (1,2), the immune clearance of certain helminths (3), and its role in

autoimmune responses leading to allergic airway inflammation (4) and colitis (5). IL-9 can be produced by mast cells, eosinophils, and type two innate lymphoid cells, but it is primarily produced by Th cells (6). These include Th2, Th17, and Treg cells (7-10); however, the principal source of IL-9 is thought to be Th9 cells (11,12), which are a newly identified subset defined by the production of IL-9 without the cytokines characteristic of other subsets. Th9 cells can be produced in vitro by the activation of naïve CD4⁺ T cells in the presence of IL-4 and TGF- β (11-13), but their role in vivo has been hard to define. Some of the best evidence for their importance has been demonstrated in mice with a T-cell specific deletion of PU.1, which is a transcription factor required for IL-9 expression (7). These mice lack Th9 cells, but the development of other Th subsets remains largely unaffected. They are resistant to airway inflammation in a model of asthma (7) and also to ulcerative colitis in a model of inflammatory bowel disease (5). Understanding the development of Th9 cells and the regulation of IL-9 expression could provide new inroads towards clinical approaches to many immune related diseases.

Transcriptional regulation plays an important role in IL-9 expression (14). Signalling through STAT6 is essential (11,12,15) and so are the transcription factors PU.1 (7,16,17), IRF4 (16), GATA3 (11), and BATF (18), whereas Id3 inhibits transcription (19). Outside of transcriptional regulation, it is not known if other mechanisms are important for IL-9 expression. MicroRNAs (miRNAs) are one such mechanism. These are double-stranded RNAs of approximately 23bp that post-transcriptionally regulate gene expression by inhibiting translation and inducing message instability (20,21). Several studies have identified their roles in Th development. First of all, the T cell specific deletion of *Drosha* or *Dicer* (RNases required for miRNA synthesis) results in a propensity towards Th1 development and a significant decrease in the number of Tregs (22-24). In addition, multiple individual miRNAs regulate various activities in Th development and function. They are thought to fine-tune the expression of genes important for development and maintenance of the stability of Th cells

(25). In this study we found that miRNAs play an important role in IL-9 expression and Th9 development and function, and we used them as a tool to discover the significance of HIF-2 α .

MATERIALS AND METHODS

Mouse strains and isolation of naïve T cells

C57BL/6 (Charles River, Kent, UK), Rag2^{-/-}, and CD4Cre *Dicer*^{lox/lox} mice were kept in a conventional specific pathogen-free facility. All the animal work was performed according to the Animals Scientific Procedures Act, UK under the animal Project Licence 70/6965. Naïve T cells (CD4⁺ CD62L^{high} CD25⁻) were isolated from pooled spleen and lymph node cells of 8-16 week old mice, first using the Dynabeads® Untouched™ Mouse CD4 cells kit (Invitrogen,), followed by biotinylated-anti-CD25 (7D4, BD Biosciences), and finally biotinylated-anti-CD62L (MEL-14, BD Biosciences), with the last two steps using streptavidin-MicroBeads (Miltenyi Biotech). Purity of cells was greater than 90% as determined by flow cytometry.

Th cell development and analysis

Naïve T cells were activated in the presence of plate-bound anti-CD3/anti-CD28 antibodies (eBioscience) with 1 μ g/ml anti-CD3 and 2 μ g/ml anti-CD28 for Th0, Th1, Th2 and induced Treg (iTreg) differentiation, or 10 μ g/ml anti-CD28 for Th17 and Th9 differentiation. T cells were differentiated into Th0 using 5 μ g/ml anti-IFN- γ (BD Bioscience) and 5 μ g/ml anti-IL-4 (BD Bioscience); Th1 using 20ng/ml recombinant-IL-12 (eBioscience) and 5 μ g/ml anti-IL-4; Th2 using 20ng/ml recombinant IL-4 (BD Bioscience) and 5 μ g/ml anti-IFN- γ ; Th17 using 2.5ng/ml recombinant TGF- β , 50ng/ml recombinant IL-6 (eBioscience), 5 μ g/ml anti-IFN- γ , 5 μ g/ml anti-IL-4, and 5 μ g/ml anti-IL-2 (BD Bioscience); Th9 using 2.5ng/ml recombinant TGF- β (eBioscience), 40ng/ml recombinant IL-4, and 10 μ g/ml anti-IFN- γ ; and iTregs using 2.5ng/ml recombinant TGF- β and 5ng/ml recombinant-IL-2 (eBioscience). Cells

were cultured for 3-4 days prior to analysis. For intracellular staining of cytokines, cells were treated with 1µg/ml each of PMA and ionomycin (Sigma) for four hours and 1µg/ml of brefeldin A (eBioscience) for two hours prior to staining. For Foxp3 staining, cells were fixed with Foxp3 fixation/permeabilization buffer (eBioscience) for 30 minutes prior to staining, and if GFP was also detected, cells were fixed with 2% paraformaldehyde for five minutes prior to fixation/permeabilization. Antibodies for flow cytometry experiments were: CD4-FITC/PE/PerCP/allophycocyanin (GK1.5), CD8α-PerCP (53-6.7), CD25-PE (PC61.5), Foxp3-allophycocyanin (FJK-16s), IFN-γ-FITC (XMG1.2), IL-17-PE and IL-9-eFlour® 660 (RM9A4) (all from eBioscience), and IL-17a-PE (TC11-18H10) and IL-4-PE (11B11) (BD Biosciences). Data were acquired using a FACS Canto II or FACS Calibur (BD Bioscience) and analysed using FlowJo software (Tree Star). Compensation and gate lines for cytokine and Foxp3 staining were set in each experiment using wild-type cells activated under Th0 polarizing conditions or as stated for the individual experiment.

Retroviral and Lentiviral production

The miR-15b/16 expression and decoy vectors were described previously (26). The expression vector was derived by cloning the genomic region encoding the miRNAs into the retroviral pMIG vector. The decoy vector was designed as described (27) to express a short RNA containing miR-15b/16 target sites. The decoy sequence was cloned into the pSIF lentiviral vector for Pol III expression. Retrovirus and lentiviruses were produced by calcium phosphate transfection of human embryonic kidney (HEK) 293T cells with helper virus vectors. Culture supernatants were harvested and used to spin-infect naïve CD4⁺ T cells that were activated overnight by plate bound anti-CD3/anti-CD28 antibodies. Cells were then differentiated into the different Th subsets as described above.

Adoptive T cell-transfer colitis model

Naïve T cells were activated and retrovirally transduced then FACS-sorted for GFP⁺ cells. These were differentiated under Th9 polarizing conditions, and 10⁶ cells were then injected into the peritoneal cavity of C57BL/6 *Rag2*^{-/-} mice. Weight was monitored every week, and mice were sacrificed after eight weeks (or when weight decreased by 20%) for analysis. Histopathological review was performed blindly as previously described (28).

Other protocols

IL-9 was measured in culture supernatants of Th differentiated cells after 48 hours of culture using murine IL-9 ELISA ready-SET-Go! second-generation kit (eBioscience). For quantitative RT-PCR (qRT-PCR), RNA was isolated from CD4⁺ T cells using miRNAeasy (for miRNAs) or mRNAeasy (for mRNAs) isolation kits (QIAGEN, Manchester, UK). For miRNA detection, cDNA synthesis and subsequent qRT-PCR with locked nucleic acid primers for specific miRNAs was performed using miRCURY LNA[™] universal reverse transcriptase microRNA cDNA synthesis and qRT-PCR kit (EXIQON). For mRNA detection, cDNA was prepared using the miRscript cDNA synthesis kit (QIAGEN). qPCR was performed using IQ[™] BioRad SYBR green master mix (BioRad, Hertfordshire, UK). Primer sequences are available upon request. For Western blotting, rabbit anti-HIF-2 α (ab179825) (Abcam) was used. For siRNA knockdown of *Epas1* expression, naïve T cells were transfected with siRNAs using Accell deliver medium (all from Dharmacon) following manufacturer's protocols. Luciferase assays were performed using the Dual-Luciferase reporter assay system (Promega) from extracts prepared from HEK293T cells transfected with a control renilla vector and the firefly reporter containing the 3' untranslated region (UTR) of *Epas1* downstream of the luciferase gene.

Statistical analysis:

Prism software (GraphPad) or Excel was used for statistical analyses to calculate mean and standard deviation values from independent experiments utilizing different pools of isolated T cells or in the case of the adoptive transfer experiments, individual mice. The student's t-test was used to calculate significance. P values equal to or less than 0.05 were considered significant.

RESULTS

*IL-9 expression is enhanced in *Dicer*^{-/-} T cells*

To analyse miRNA control of IL-9 expression, we utilized mice with a T-cell specific deletion of *Dicer* containing a floxed allele of *Dicer* and a CD4-Cre transgene (23). As a starting point, IL-9 FACS staining was optimized utilizing naïve Th cells (CD4⁺ CD62L^{high} CD25⁻) activated under different polarization conditions. Only under Th9 polarizing conditions were substantial numbers of IL-9-expressing cells observed (Sup. Fig. 1). Therefore, we used these staining conditions in subsequent experiments. Next, IL-9 expression was examined in the CD4⁺ population of spleen and lymph node cells (or isolated CD4⁺ cells) that were activated by PMA and ionomycin and that were from either CD4-Cre *Dicer*^{+/-lox} or CD4-Cre *Dicer*^{lox/lox} mice. In the absence of miRNAs, there was approximately a five-fold increase in the percentage of IL-9-producing cells on average, whereas consistent with our previous results (23), there was a decrease in the numbers of Foxp3⁺ cells (Fig. 1A). Substantiating this increase in IL-9 expressing cells was a twelve-fold increase in *Il9* mRNA in *Dicer*^{-/-} ex vivo isolated and unstimulated CD4⁺ CD25⁻ cells (Fig 1A, far right). Since Th9 cells are the main producers of IL-9 (11,12), we examined the importance of miRNAs on Th9 differentiation in vitro by activating naïve Th cells under Th9 polarizing conditions using TGF-β and IL-4. This results in reduction in the number of Foxp3⁺ cells that would be induced by TGF-β alone, and it produces a population of IL-9⁺ Foxp3⁻ cells classically defined as Th9 (12). *Dicer*^{-/-} Th cells had

a significant decrease in the percentage of Foxp3⁺ cells and a significant increase in the percentage of IL-9⁺ Foxp3⁻ cells, (Fig. 1B and Sup. Fig. 2). This increase in IL-9 expressing cells was also substantiated by an approximately two-fold increase in *Il9* mRNA in *Dicer*^{-/-} cells (Fig. 1B far right). Therefore, miRNAs appear to suppress Th9 differentiation. Interestingly, *Dicer*^{-/-}, ex vivo isolated Tregs (CD4⁺ CD25⁺), which are primarily thymus-derived Tregs, also had approximately a two-fold increase in the percentage of IL-9-producing cells and an approximately 3.5-fold increase in *Il9* mRNA (Fig. 1C). Therefore, miRNAs also appear to suppress IL-9 expression in Tregs.

To examine if miRNAs suppress IL-9 production in other Th subsets, naïve Th cells were activated under different polarization conditions, and the level of IL-9 production was measured in culture supernatants (Fig. 2A). *Dicer*^{-/-} Th cells had an increase in IL-9 production under all polarizing conditions tested, particularly in Th2 and iTreg conditions where the increase was to the same level found in Th9 cells. Therefore, miRNAs play a general role in suppressing IL-9 expression in all Th polarization conditions, and they are most important in Th2 and iTreg conditions. To determine if miRNAs suppress the expression of IL-9 within each subset or alternatively prevent the differentiation towards a Th9 like phenotype, we examined IL-9 expression in combination with a signature marker of a specific Th subset (IFN- γ for Th1, IL-4 for Th2, IL-17 for Th17, and Foxp3 for Tregs). Consistent with previous reports (22,23,29), loss of miRNAs resulted in increased production of IFN- γ in Th0 and Th1 conditions and increased IL-4 in Th2 conditions, whereas there was a slight loss of IL-17 expression under Th17 conditions and a significant decrease of Foxp3 expression in induced Tregs (iTregs) (Fig. 2B). The increase in IL-9 expression in all subsets but iTregs occurred in both populations of cells expressing or not expressing the signature cytokine. Therefore, miRNAs appeared to be important regulators of IL-9 expression within these subsets but also important for inhibiting differentiation into a Th9-like phenotype. In contrast, the increase in IL-9 expression in iTreg induction occurred primarily in cells not expressing Foxp3.

Therefore, miRNAs appeared to be solely important for the lineage choice in these conditions but not for the regulation of IL-9 expression in cells with an iTreg phenotype, which was unlike ex vivo-isolated Tregs that expressed more IL-9 in the absence of miRNAs.

miR-15b/16 suppresses IL-9 production in Th9 cells

To begin understanding miRNA regulation of IL-9 expression, we first wanted to identify individual miRNAs that could function in suppression. Subsequently, relevant targets could be determined whose regulation impacted IL-9 expression. Because loss of miRNAs had an opposite effect on the differentiation of iTregs and Th9 cells, we decided to test the function of miRNAs that we previously found to be important for iTreg induction (26) and determine if they could additionally suppress Th9 differentiation. miR-15b and miR-16 were two of these. They are highly related miRNAs encoded on the same primary transcript that target the same messages. They enhance iTreg induction through their suppression of mTOR and Rictor, which inhibits mTOR signalling and directs cells into a Treg differentiation pathway. We previously found that miR-15b and miR-16 were more abundantly expressed in iTregs than in naïve T cells or those activated under Th0, Th1, Th2, or Th17 polarizing conditions, which was consistent with their importance in iTreg induction (26). Here we show those data again and add the miR-15b and miR-16 expression levels in Th9 cells, which were also much lower than iTregs (Fig. 3A). Therefore, miR15b/16 might regulate IL-9 expression in iTregs, but they would unlikely be important in other subsets. Nevertheless, to determine if miR-15b/16 were capable of suppressing IL-9 expression, we examined if their overexpression could inhibit IL-9 expression in Th9 polarization conditions. Overexpression was achieved utilizing a GFP-expressing retrovirus that contained the genomic sequence encoding both miRNAs. This or a control, empty retrovirus was transduced into naïve CD4⁺ T cells after their activation, which gave on average twice the expression level of these miRNAs in iTregs (Sup. Fig. 3). Cells were subsequently activated under Th9 polarizing conditions, and IL-9 expression was examined in

transduced, GFP⁺ cells. miR15b/16 overexpression resulted in a significant reduction in the percentage of IL-9-producing cells and the level of IL-9 production (Fig. 3B and C). In contrast, miR-15b/16 overexpression did not have an effect on the low numbers of cells expressing IL-9 in Th1, Th2, Th17, or iTreg polarizing conditions (Fig. 3D-G). Likewise, miR-15b/16 overexpression did not affect the development of Th1, Th2, or Th17 cells as measured by the expression of signature cytokines, but consistent with our previous findings, it did enhance iTreg induction (26). Therefore, miR15b/16 appear to be capable of regulating Th9 and iTreg differentiation but not other subsets.

miR-15b/16 inhibits the inflammatory response of Th9 cells in vivo

Because Th9 cells are important in autoimmunity, we examined if miR-15b/16 could inhibit their inflammatory response. In vitro-derived Th9 cells will produce an autoimmune response in the colon when they are adoptively transferred into *Rag2*^{-/-} mice (11). Therefore, we tested if miR-15b/16 overexpression could inhibit this activity. Naïve CD4⁺ CD25⁻ T cells were activated then transduced with control or miR-15b/16 expressing retroviruses. GFP⁺ cells were sorted then further activated under Th9 polarizing conditions before their adoptive transfer into *Rag2*^{-/-} mice. miR-15b/16 overexpression significantly inhibited weight loss (Fig. 3A) and reduced the resultant colitis as measured by colon thickness (Fig. 4B). Analysis of GFP⁺ cells recovered from the spleen revealed that miR-15b/16 overexpression reduced the number of IL-9-producing CD4⁺ cells (Fig. 4C). However, miR-15b/16 overexpression also resulted in an increase in the number of Tregs (Fig. 4D) so it was uncertain if the reduced inflammatory response was due to the reduction in Th9 cells or an increase in Tregs. Nevertheless, the effects of miR-15b/16 overexpression on IL-9 expression in Th9 polarization observed in vitro were maintained in vivo over the time course of this experiment.

Epas1, encoding the hypoxia transcription factor HIF-2 α , can be regulated by miR-15b/16

Because miR-15b/16 could regulate the expression of IL-9, and because these miRNAs are highly expressed only in Tregs, we hypothesized that miR-15b/16 might be important for regulating IL-9 expression in Tregs. Therefore, we searched for potential target genes using target prediction algorithms (30). Because true target genes would most likely be more highly expressed in *Dicer*^{-/-} Tregs, potential targets of miR-15b/16 were manually examined for those that were more highly expressed in *Dicer*^{-/-} compared to *Dicer*^{+/+} Tregs using gene expression array data (31). *Epas1* was one gene found in this analysis, which encodes the hypoxia inducible factor HIF-2 α . HIF-2 α or its related gene product, HIF-1 α , associates with HIF-1 β (also known as the aryl hydrocarbon receptor nuclear translocator ARNT). These heterodimers act as transcription factors to regulate gene expression for cell survival in low oxygen environments (32). HIFs play important roles in cancer biology due to the hypoxic conditions that develop as tumors grow (33). Originally, the HIF- α subunits were thought to be interchangeable, but recent evidence has found differences in their function (33). HIF-1 α plays a complicated role in Th development in that it has been found to be important for the development of Th17 cells over Tregs (34,35), but it is also important for Treg function (36). In contrast, virtually nothing is known about the function of HIF-2 α in Th development. Therefore, its regulation by miRNAs was examined.

Epas1 RNA was abundantly expressed in Th9-polarized cells, which was consistent with a role in IL-9 expression. However, it was also abundant in Th2-polarized cells (Fig. 5A), but whereas the highest RNA level was found in Th2-polarized cells, the highest protein level was found in Th9-polarized cells (Fig. 5C). In either case, it was less expressed in Tregs and other Th subsets. Therefore, its abundant expression in Th9 cells was consistent with a role in IL-9 expression. However, its expression in Th2 cells indicated that it most likely would not be sufficient.

The expression of *Epas1* was regulated by miRNAs, as there was a significant increase in mRNA levels in ex vivo-isolated CD4⁺ conventional T cells (Tcon) (CD25⁻) and Tregs (CD25⁺) that lacked miRNAs (Fig. 5B). Likewise, HIF-2 α protein levels were increased in *Dicer*^{-/-} T cells that were activated under all polarization conditions tested (Fig. 5C). Therefore, expression of HIF-2 α could be regulated by miR-15b/16 in Tregs, but other miRNAs would most likely be important in other subsets because as stated above, miR-15b/16 are only abundantly expressed in Tregs. Nevertheless, we examined regulation of HIF-2 α expression by miR-15b/16. The activity of a luciferase reporter gene containing the *Epas1* 3'UTR was suppressed by the co-expression of miR-15b/16, and this suppression was significantly reduced if the predicted miR-15b/16 target site was mutated (Fig. 5D). In addition, expression of *Epas1* RNA and HIF-2 α protein was suppressed in Th9-polarized cells by the overexpression of miR-15b/16 (Fig. 5E). Therefore, miR15b/16 can regulate the expression of *Epas1*.

HIF-2 α regulates the development of Th9 cells and iTregs

To test the importance of HIF-2 α in the development of Th9 cells, the effect of altering its expression was analysed. Transfection of an siRNA against *Epas1* reduced the percentage of IL-9-producing cells when activated under Th9 polarizing conditions, whereas it enhanced the production of Foxp3⁺ cells (Fig. 6A). The observed decrease in the numbers of IL-9 expressing cells was substantiated by the reduced production of IL-9 in culture supernatants from these cultures (Fig. 6A). In contrast to the siRNA knockdown, overexpression of HIF-2 α using a retrovirus containing the *Epas1* cDNA, enhanced the percentage of IL-9-producing cells and decreased the production of Foxp3⁺ cells (Fig. 6B). Overexpression of *Epas1* also inhibited the induction of iTregs in iTreg conditions, but as in the Foxp3⁺ cells in Th9 polarization conditions, it did not increase IL-9 expression. Therefore, expression of HIF-2 α is also not sufficient for IL-9 expression in iTregs. Other regulatory factors specific to Th9 cells must also be required. To test the importance of miR-15b/16 suppression of HIF-2 α expression in Tregs, naïve T cells were activated and transduced with a lentivirus expressing

a miR-16 decoy that acted as a competitive inhibitor of miR-15b/16. These cells were then differentiated under iTreg conditions. Inhibiting the high levels of miR-16 in Tregs increased the expression of *Epas1* mRNA and HIF-2 α protein (Fig 6D), and consistent with our previous results (26) it inhibited iTreg induction, but ironically it did not increase IL-9 expression (Fig. 6E). Therefore, the loss of miR15b/16 expression in *Dicer*^{-/-} iTregs is not sufficient for the developmental changes that lead to some cells with a Th9 like phenotype. Other miRNAs must be important in iTregs and also for regulation of IL-9 expression in other subsets. Nevertheless, miR15b/16 appear to be one means of suppressing the expression of HIF-2 α in iTregs and preventing its inhibitory effects on iTreg induction.

DISCUSSION

In this report we have demonstrated that miRNAs regulate the expression of IL-9 in Th cells. Two highly related miRNAs, miR-15b and miR-16, were found capable of suppressing IL-9 expression when overexpressed in Th9 cells. These miRNAs were able to regulate the hypoxia transcription factor gene *Epas1* (encoding HIF-2 α), which was found to be important for IL-9 expression in Th9 cells.

This study demonstrated the utility of examining miRNA function to identify new regulatory pathways in Th development and function. However, the irony is that the miRNAs analysed (miR-15b/16) are probably not directly relevant for regulating IL-9 expression in vivo because 1) they could only suppress IL-9 expression when they were overexpressed in Th9 cells; 2) they are not abundantly expressed in Th0, Th1, Th2, and Th17 cells, in which IL-9 expression is low so they are unlikely to be important regulators in these subsets; and 3) in iTregs where miR-15b/16 are abundantly expressed, blocking their function did not induce IL-9 expression. Therefore, other miRNAs must be responsible for the increased IL-9 expression observed in *Dicer*^{-/-} T cells. Nevertheless, miR-15b/16 regulation of HIF-2 α expression in iTregs appears to be one mechanism of suppressing the expression of HIF-2 α

and preventing its inhibitory function on iTreg induction. Therefore, HIF-2 α appears to be an additional target of miR-15b/16 in iTregs that explains the importance of these two miRNAs in iTreg induction. All these findings are summarized in the diagram in figure 7.

The suppression of iTreg induction by HIF-2 α and its importance for the expression of IL-9 in Th9 cells adds new information to the function of the HIF proteins in Th development. As mentioned above, HIF-1 α plays a complicated role in Th development with many questions regarding its functions (37). HIF-1 α is reported to enhance Th17 over Treg development (34,35), but it is also required for Treg function (36). In Th17 cells, HIF-1 α associates with the transcription factor ROR γ t to regulate transcription of genes important for Th17 development. It also inhibits Treg development through its association with Foxp3, which targets it for degradation through the proteasome (35). However, hypoxia also directly activates FoxP3 expression and the induction of Tregs (36). Therefore, the details of HIF-1 α function need further understanding. HIF-2 α appears to regulate the differentiation between Th9 and Tregs analogous to HIF-1 α in the Th17/Treg axis. However, the mechanisms of HIF-2 α activating IL-9 expression and suppressing Treg induction will need to be explored. With IL-9 expression, clearly HIF-2 α is not sufficient by itself because its relevantly abundant expression in Th2 cells or its overexpression in iTregs does not lead to high expression of IL-9. Other factors specific to Th9 cells must also be required. Finally, both HIF-1 α and HIF-2 α are regulated by oxygen levels through hydroxylation of key proline residues, which leads to their degradation by the proteasome. In T cells HIF-1 α is also stabilized under normal oxygen conditions by TCR stimulation or IL-6 signalling through STAT3 (35,38). Therefore, regulation of HIF protein expression plays an important role in the diverse environments encountered by T cells in vivo.

Regulation of IL-9 expression is controlled by many mechanisms, and this work has illustrated the importance of miRNAs and HIF-2 α . Therefore, it will be important to understand how all these combine to regulate IL-9 expression in an immune response.

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AUTHOR CONTRIBUTIONS

YS and BC planned the experiments, and YG performed the vast majority. BC constructed some of the recombinant vectors and OG scored the pathology of the adoptively transferred mice. FL provided financial support. YS and BC wrote the manuscript with editing by OG and FL.

CONFLICT OF INTEREST

The authors have no conflicting interest in the publication of this work.

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FIGURE LEGENDS

Figure 1. miRNAs suppress IL-9 expression in Th cells. **A.** Spleen and lymph node cells from CD4 Cre *Dicer*^{+/lox} or CD4 Cre *Dicer*^{lox/lox} mice were activated by PMA and ionomycin for four hours with brefeldin A. The analysis of Foxp3 APC and either IL-9 PE or IL-9 FMO control is shown on the CD4⁺ population of activated (as determined by SSC and FSC) cells. To the right, the mean and standard deviation values are shown from seven independent experiments (using either spleen and lymph node cells or isolated CD4⁺ cells) indicating a significant increase in IL-9 expressing cells when *Dicer* is absent (**p=0.002). Substantiating this and shown at the far right was an increase in the relative level of *Il9* mRNA in ex vivo-isolated CD4⁺ CD25⁻ cells (as measured by qRT-PCR and normalized to GAPDH) (*p=0.02 from 5 independent experiments). **B.** Naïve CD4⁺ T cells were activated under Th9-polarization conditions, and IL-9 and Foxp3 expression was analysed by FACS as in A. There was a significant increase in IL-9 expressing cells when miRNAs were absent (*p=0.02 from four independent experiments). Likewise, there was a significant increase in the relative level of *Il9* mRNA (*p=0.02 from five independent experiments). **C.** Isolated Tregs (CD4⁺ CD25⁺) also displayed an increase in IL-9 expression in *Dicer*^{-/-} cells (*p=0.01 for FACS and *p=0.04 for *Il9* mRNA from three or five independent experiments, respectfully).

Figure 2. miRNAs suppress IL-9 expression in all Th subsets. **A.** Naïve CD4⁺ cells containing (*Dicer*^{+/+}) or lacking (*Dicer*^{-/-}) miRNAs were activated under the indicated polarization conditions for 48 hours, and the level of IL-9 was determined by ELISA from culture supernatants. Values are from three independent experiments. Cells lacking miRNAs expressed significantly more IL-9: Th0 (***p=0.0003), Th1 (*p=0.03), Th2 (*****p=0.000008), Th9 (*p=0.05), Th17 (**p=0.002) and iTregs (***p=0.0003). **B.** Representative profiles from 3-4 independent experiments showing expression of signature cytokines (or Foxp3 for iTregs) and IL-9 for indicated polarization conditions.

Figure 3. Overexpression of miR-15b/16 suppresses the expression of IL-9 during Th9 polarization but has no effect on Th1, Th2, or Th17 polarization. **A.** Relative miRNA levels of miR15b and miR-16 normalized to 5S rRNA (as determined by qRT-PCR) from naïve CD4⁺ T cells activated under the indicated polarization conditions. Th9 levels are added for comparison to data from our previous report (26) of levels in other subsets. **B-G.** T cells transduced with a control or miR 15b/16 overexpressing retrovirus were activated under the indicated polarization conditions. Th differentiation in the GFP⁺ cell population was measured by the percentage of cells expressing the signature cytokine (B, D-F) or Foxp3 for iTregs (G). A representative experiment is on the left, and the mean and standard deviation values are on the right for four independent experiments. Only in Th9 polarized cells was the percentage of IL-9 expressing cells significantly different (*p=0.02). **C.** mR-15b/16 overexpressing cells produce less IL-9 during Th9 polarization, as measured by ELISA from culture supernatants. Data are from three independent experiments (**p=0.001).

Figure 4. miR-15b/16 overexpression suppress the inflammatory response of Th9 cells.

A. & B. Naïve CD4⁺ cells were activated then transduced with control or miR-15b/16 expressing retroviruses. GFP⁺ cells were sorted then differentiated under Th9 polarization conditions and adoptively transferred into *Rag2*^{-/-} mice as described in Materials and Methods. Weight loss (A) was significantly inhibited when Th9 differentiated cells overexpressed miR-15b/16 (**p=0.002), as was the resultant colitis (B) as measured by colon thickness (*p=0.03). **C. & D.** Spleen cells were isolated from the above mice and activated by anti-CD3 and anti-CD28 then GFP⁺ cells (which derived from the original adoptively transferred cells) were analysed for expression of IL-9 (C) or Foxp3 (D) in combination with CD4. A representative experiment is shown on the left and the mean and standard deviation values on the right, which show a significant decrease in IL-9 expressing cells (**p=0.005) and

a significant increase in Foxp3 expressing cells (**p=0.006). Data are derived from four mice per group.

Figure 5. HIF-2 α is regulated by miR-15b/16 overexpression. **A.** *Epas1* mRNA levels normalized to GAPDH (as measured by qRT-PCR) and relative to naïve T cells are highest in T cells differentiated under Th2 and Th9 conditions. Thymus-derived Tregs (tTregs) are ex vivo isolated Tregs. Data are from 4 independent experiments. **B.** *Dicer*^{-/-} T cells have significantly increased *Epas1* mRNA levels in both Tcon (CD25⁻) (**p=0.002) and Tregs (CD25⁺) (**p=0.003). Data are from 4 independent experiments. **C.** HIF-2 α levels are highest in Th9 and Th2 differentiated cells, and levels increase in all helper subsets when miRNAs are absent. On the left is a representative Western blot, and on the right are the values normalized to GAPDH and relative to *Dicer*^{+/-} Th0 cells as determined by densitometry from 4 independent experiments. **D.** *Epas1* contains a miR15b/16 target site in its 3' UTR. The sequence of the region containing the miR-15b/16 target site in a luciferase reporter vector with the *Epas1* 3'UTR is shown as well as the sequence of a mutated reporter. Below is the sequence of miR-15b showing the homology through the seed sequence. When the wild type reporter was transfected into HEK 293T cells, it was significantly repressed by the co-expression of miR15b/16 (****p=0.00003), and suppression was significantly reduced with the reporter containing the mutated miR-15b/16 target site (**p=0.002). **E.** Overexpression of miR-15b/16 suppressed *Epas1* mRNA and HIF-2 α protein expression in Th9 differentiated cells. mRNA levels are derived from 3 independent experiments (*p=0.04), and the Western blot is representative of two independent experiments with the level of HIF-2 α relative to GAPDH in this blot (as determined by densitometry) shown between.

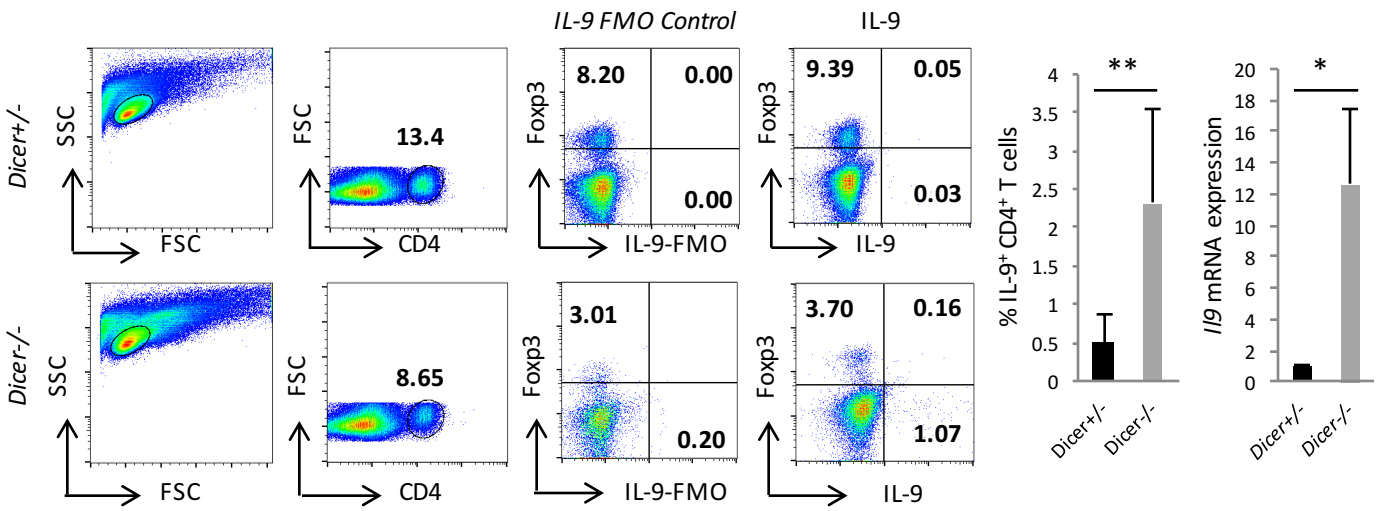
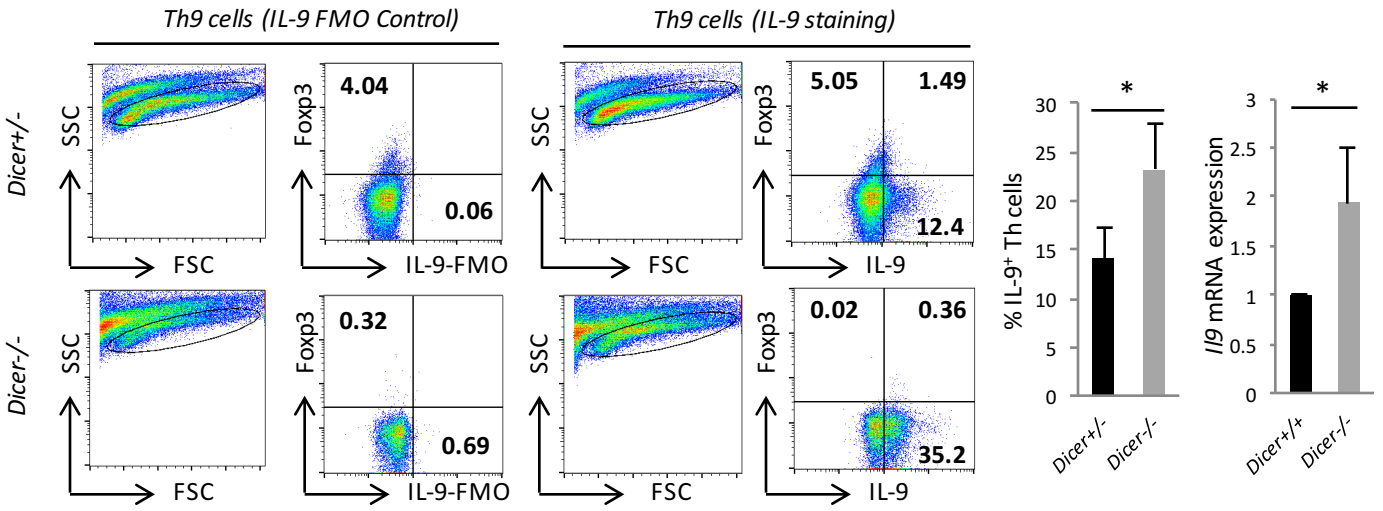
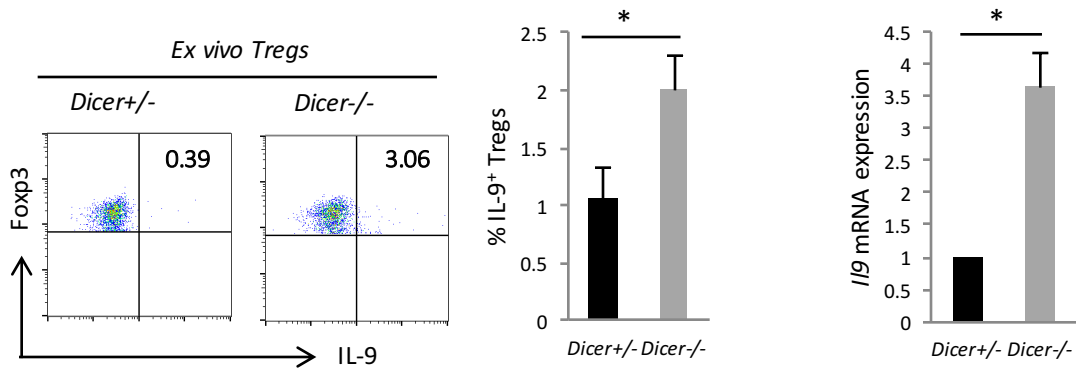
Figure 6. HIF-2 α enhances Th9 development and inhibits iTreg induction. Naïve CD4⁺ T cells were activated and transfected with control or *Epas1* siRNAs (A) or transduced with

control or *Epas1* expressing retroviruses (B, C) or with a control or a miR-16 decoy expressing lentivirus (D, E). Cells were then activated under Th9 polarization (A, B) or iTreg induction (C, D, and E) conditions. **A.** An *Epas1* siRNA inhibits Th9 differentiation (*p=0.02) and also increases the number of Foxp3⁺ IL-9⁻ cells in these conditions. It also inhibits the production of IL-9 in culture supernatants as measured by ELISA (*p=0.02). The levels of HIF-2 α as measured in a Western blot are displayed on the far right. **B & C.** Overexpression of *Epas1* enhances Th9 differentiation (*p=0.01) and inhibits the induction of iTregs (**p=0.004). **D.** Expression of a miR-16 decoy during the induction of iTregs enhances the expression of *Epas1* (as measured by qRT-PCR and normalized to GAPDH *p=0.02) and also HIF-2 α (as measured by Western blot and also normalized to GAPDH). **E.** Despite the inhibition of iTreg induction by the miR-16 decoy (*p=0.01), it does not increase IL-9 expression in iTregs. For FACS data, a representative experiment is shown with the mean and standard deviation values from four to six experiments. The ELISA and *Epas1* mRNA data are derived from four or three independent experiments respectfully, and the HIF-2 α Western blots are representative of two independent experiments with similar results. The relative levels of HIF-2 α normalized to GAPDH for these blots as determined by densitometry is shown.

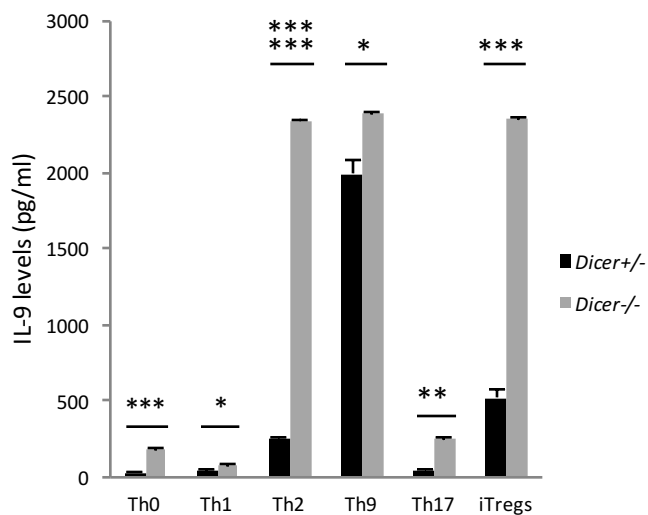
Figure 7. Summary diagram of miRNA and HIF-2 α regulation of Th9 differentiation and IL-9 expression. miR-15b/16 play important regulatory roles in iTregs. Our previous work demonstrated that these miRNAs enhance iTreg induction through suppressing mTOR signalling. In this work we have found that they also enhance iTreg induction through suppressing HIF-2 α expression, which like HIF-1 α , inhibits iTreg induction. In contrast, HIF-2 α is important for Th9 differentiation but not sufficient for IL-9 expression in other subsets. Finally, miR-15b/16 do not appear to be the important miRNAs for regulating IL-9 expression in all Th subsets. Therefore, as yet undefined miRNAs must be involved in suppressing Th9 differentiation and IL-9 expression in all Th subsets.

A

PMA/ionomycin activated spleen and lymph node cells gated on CD4⁺ T cells

**B****C**

A



B

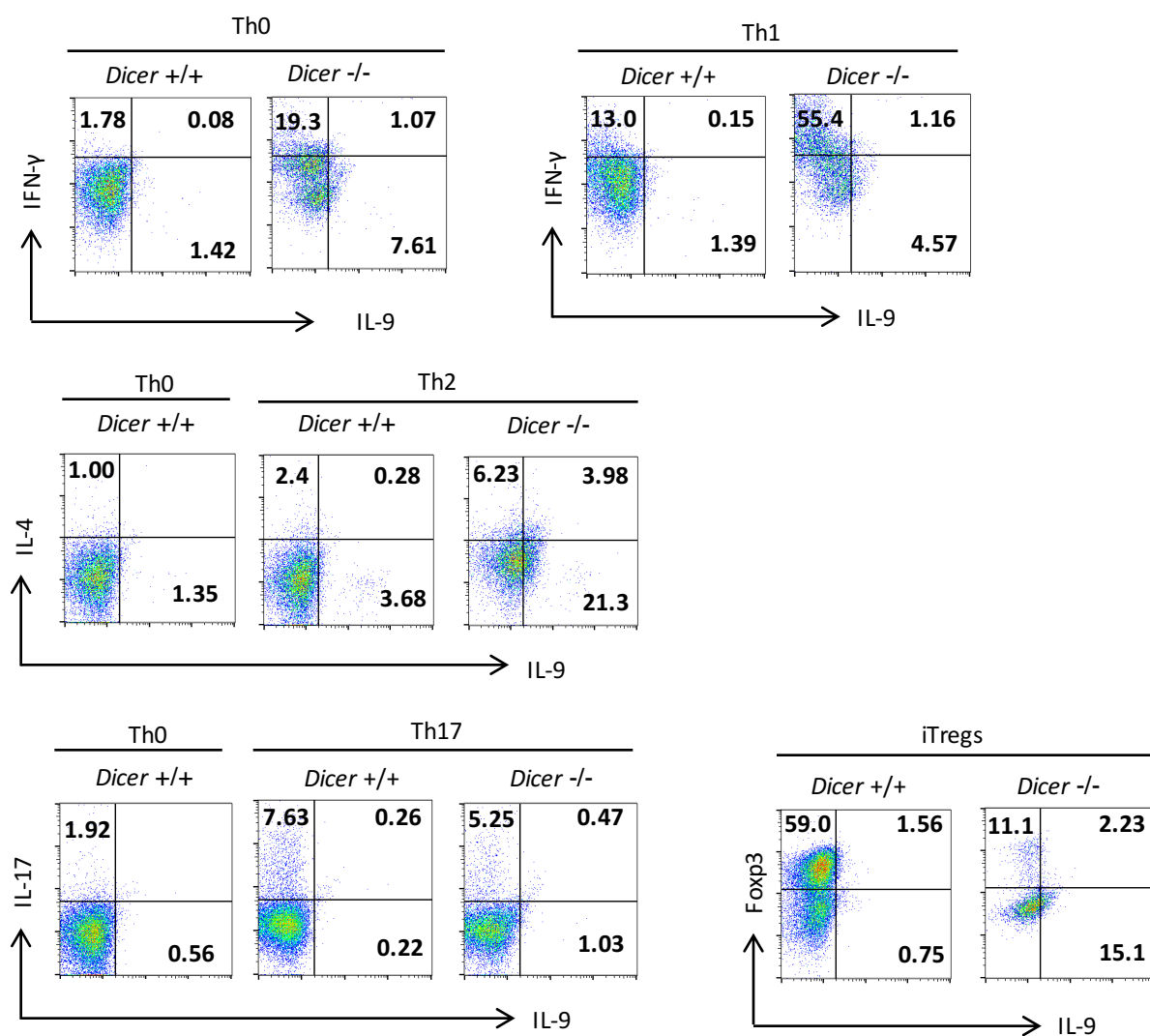


Fig. 2

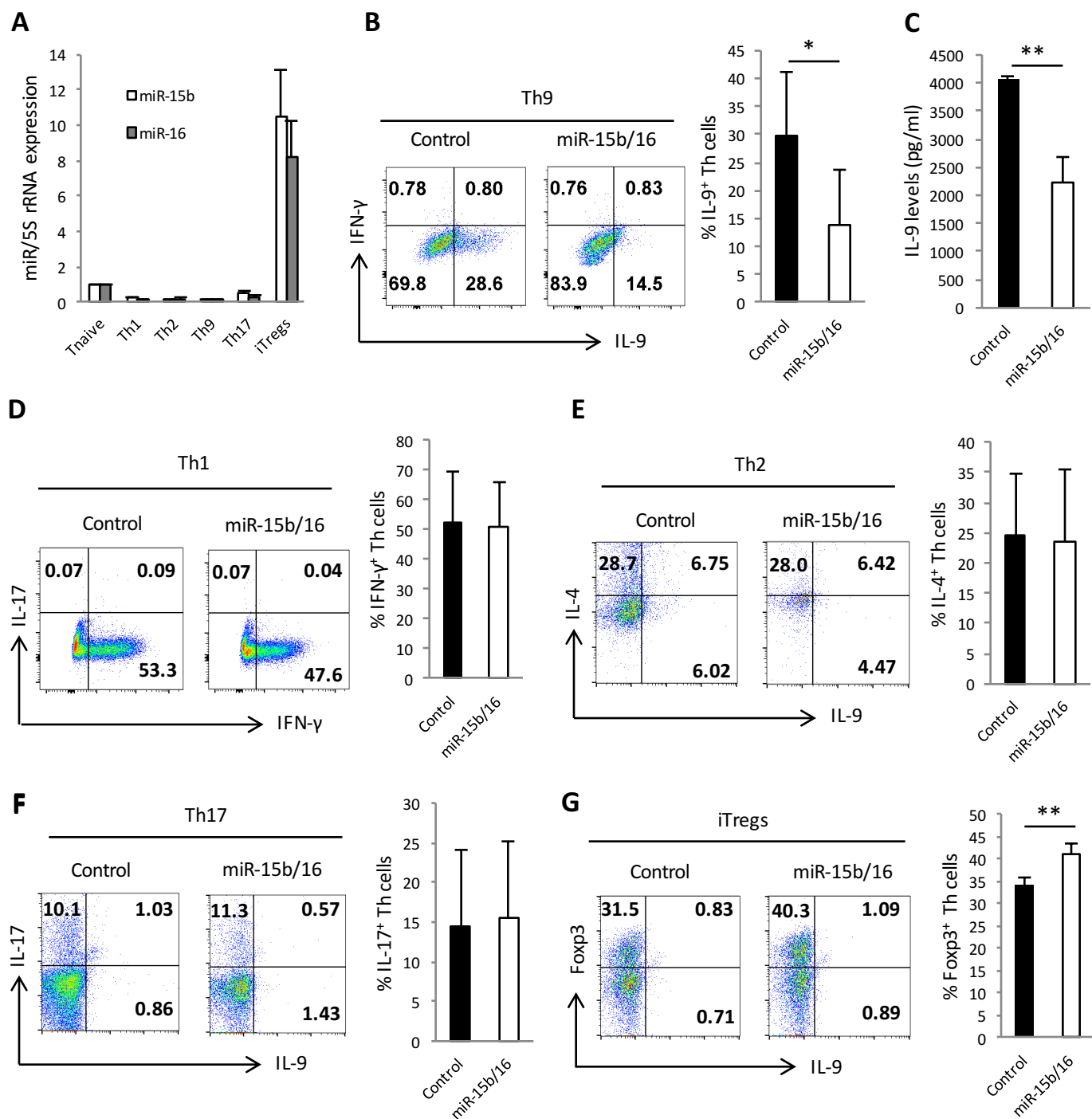


Fig. 3

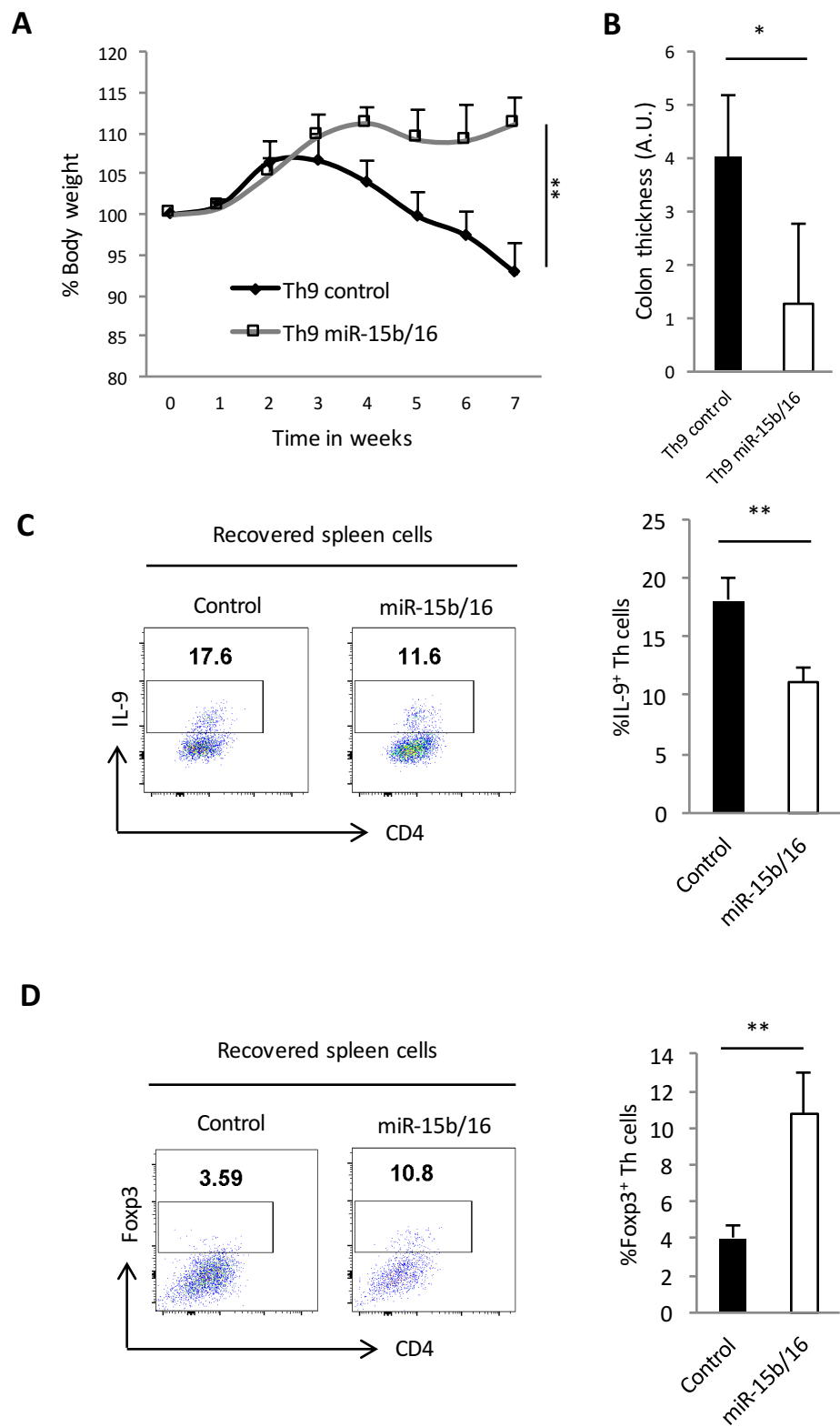


Fig. 4

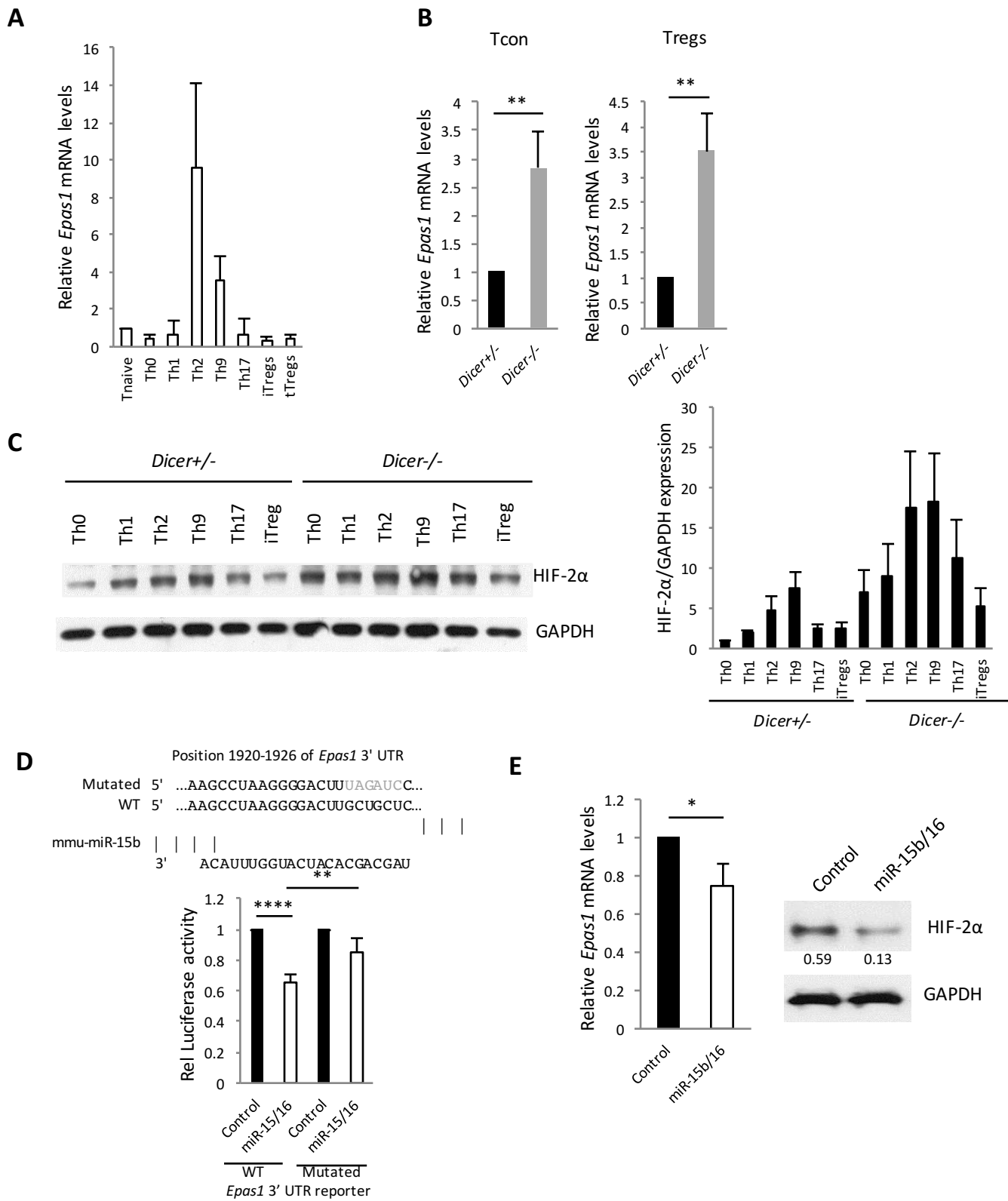


Fig. 5

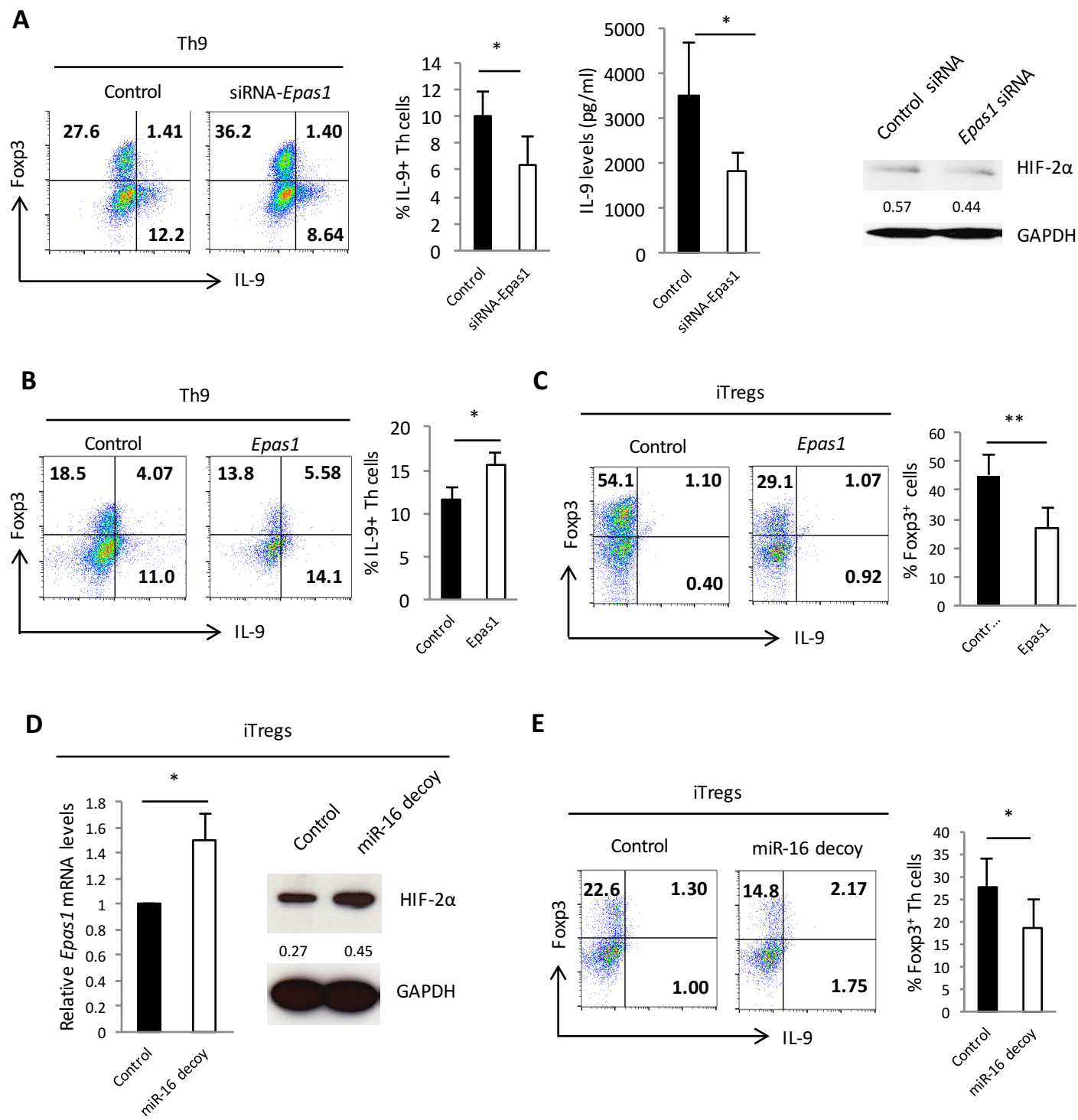


Fig. 6

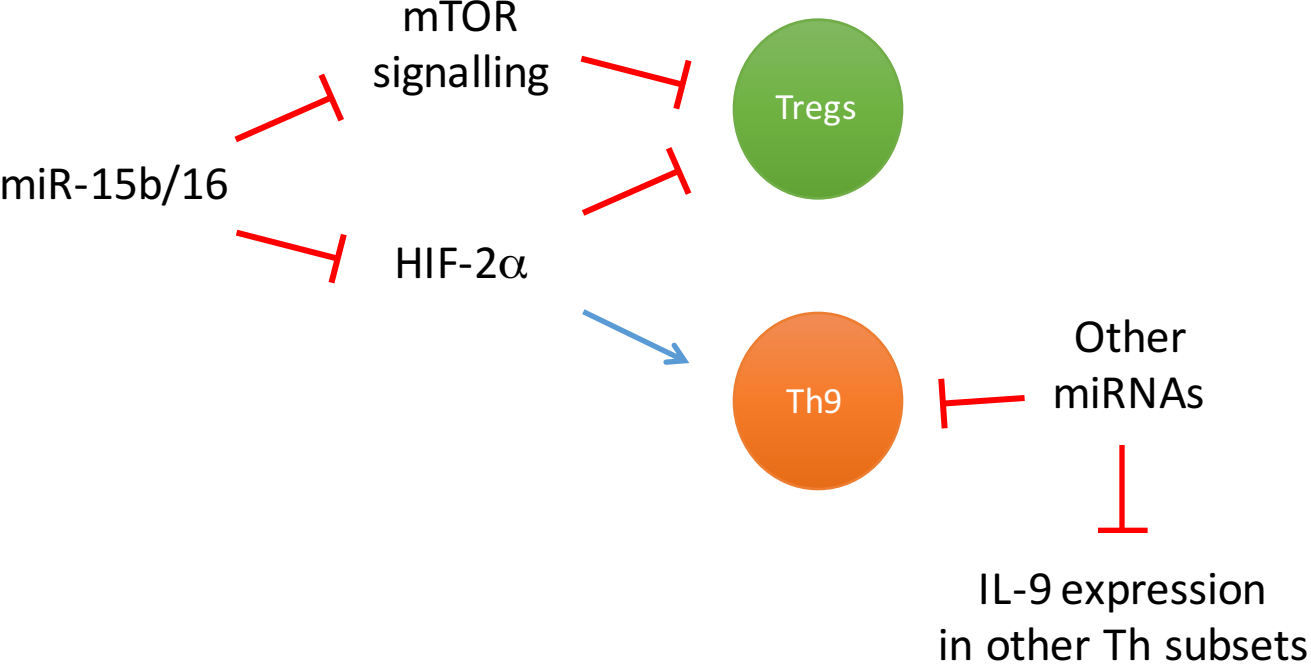
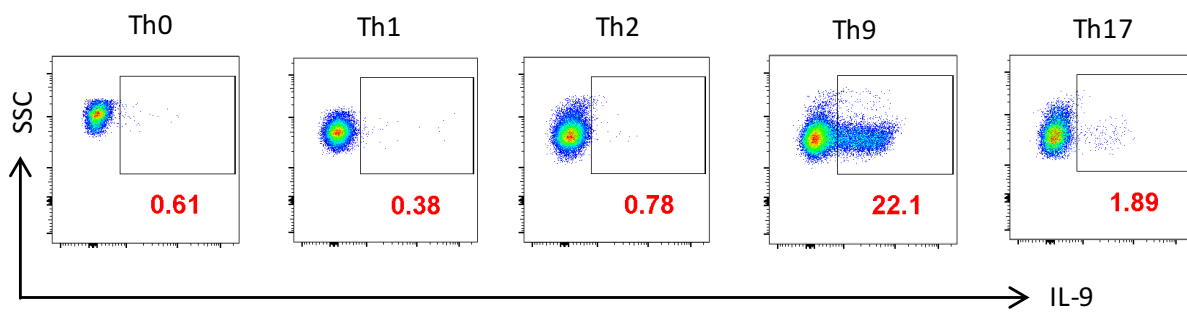
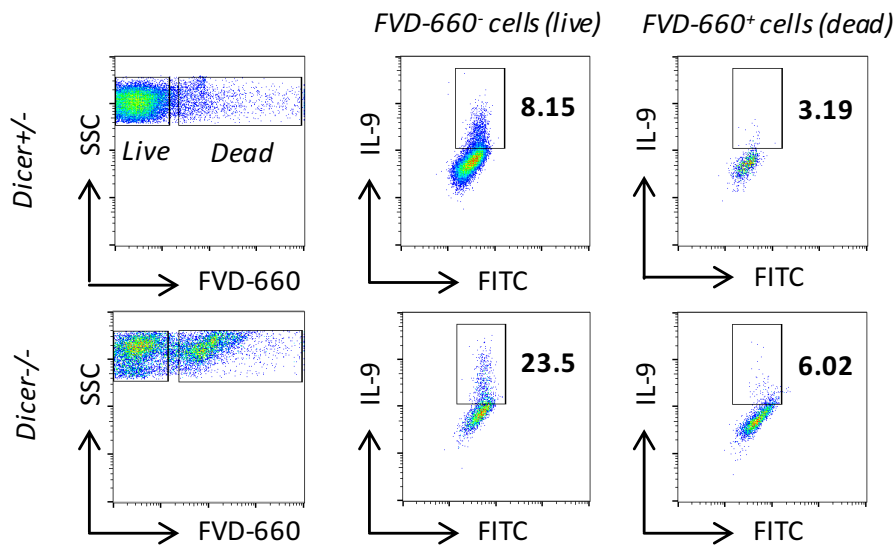
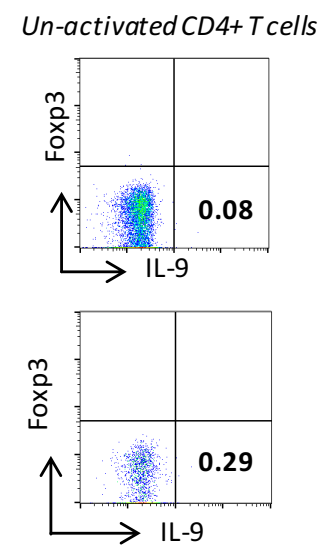


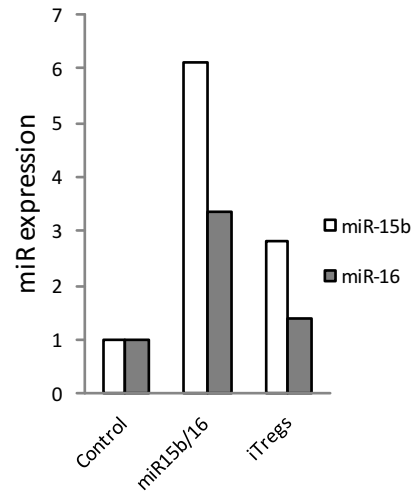
Fig. 7



Supplemental Figure 1. Verification of IL-9 detection in FACS staining. Naïve CD4⁺ T cells were activated under the indicated polarization conditions then cells were fixed and stained for IL-9 expression. A representative experiment is shown demonstrating the greatest expression in Th9 cells.

A**B**

Supplemental Figure 2. Live/dead and unstimulated cell controls for IL-9 detection in Th9-differentiated T cells. **A.** Naïve *Dicer*^{+/−} and *Dicer*^{−/−} Th cells were activated under Th9 polarization conditions, and cells were stained with IL-9 PE and the Fixable Viability Dye eFlour660 (FVD-660) (eBioscience), which can be visualized in the APC channel. The IL-9 analysis is shown against the open FITC channel from both the live and dead populations. **B.** Staining of Foxp3 APC and IL-9 PE from un-activated cells of the same preparation used for A.



Supplemental Figure 3. Expression levels of miR-15b and miR-16 in retrovirally transduced T cells relative to iTregs. The relative expression of miR-15b and miR-16 was determined by qRT-PCR from T cells transduced by a miR-15b/16 retrovirus or from those induced to become iTregs. Because of the difficulty of measuring overexpression in the mixed population of transduced and untransduced cells, the comparison with iTregs was made between cells with a similar transduction efficiency (~30%) and iTreg conversion frequency (~40%). The mean values from two independent experiments with similar results are shown.