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MicroRNA Regulation of Helper T Cell Cytokine Production and Differentiation

Author
Steiner, David F.

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MicroRNA Regulation of Helper T Cell Cytokine Production and Differentiation

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David F. Steiner

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Contributions of Co-authors to the Presented Work:

Chapter 2 of this dissertation was published as "MicroRNA-29 regulates T-box transcription factors and interferon-γ in helper T cells" in *Immunity*. 2011 Aug 26;35(2):169-81. The co-authors on this paper were Molly F. Thomas[1], Joyce K. Hu[2], Zhiyong Yang[1], Joshua E. Babiarz[3], Christopher D.C. Allen[1], Mehrdad Matloubian[2], Robert Blelloch[3], K. Mark Ansel[1]. Molly F. Thomas generated the deep sequencing results utilized in this study with assistance in data analysis from Joshua E. Babiarz. Joyce K. Hu and Mehrdad Matloubian assisted with and supervised LCMV experiments, especially those involving P14 CD8 cells. Zhiyong Yang and Christopher D.C. Allen performed early experiments to optimize and validate technical aspects of the transfection system critical to this work. Robert Blelloch provided *Dgcr8*$^{fl/fl}$ mice used in this study and provided critical reading of the manuscript. K. Mark Ansel supervised this work.

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[1] Sandler Asthma Basic Research Center, and Department of Microbiology & Immunology, University of California, San Francisco.

[2] Division of Rheumatology, Department of Medicine, and Rosalind Russell Medical Research Center for Arthritis, University of California, San Francisco.

[3] The Eli and Edythe Broad Center of Regeneration Medicine and Stem Cell Research, Center for Reproductive Sciences, and Department of Urology, University of California, San Francisco.

MicroRNA Regulation of Helper T Cell Cytokine Production and Differentiation

David F. Steiner

ABSTRACT

Helper T cells play a critical role in the maintenance and coordination of a healthy immune system. Upon stimulation, naïve helper T cells transition into functional, effector cells that proliferate, migrate, and actively secrete cytokines to help direct an immune response. Importantly, programmed differentiation of helper T cells into unique subsets of effector cells allows for functionally different responses to different immune challenges. The actual process of helper T cell polarization involves many regulated changes in gene expression and represents both an important aspect of immune regulation and a useful system for studying cell differentiation in general.

MicroRNA (miRNA)-deficient helper T cells exhibit abnormal differentiation, cytokine production and decreased proliferation. However, the contributions of individual miRNAs to this phenotype remain poorly understood. We developed and utilized a screening strategy to assay miRNA function in primary T cells and identified individual miRNAs that regulate the cytokine production and proliferation of these cells.

We first focused on interferon (IFN)-γ production and found that microRNA-29 (miR-29) largely corrected the aberrant IFN-γ expression of miRNA-deficient cells. Repression of IFN-γ by miR-29 involved direct targeting of both T-bet and Eomes, two transcription factors known to induce IFN-γ production. These two transcription factors were elevated in miRNA-deficient cells and were also upregulated following miR-29 inhibition in wildtype cells. These results demonstrate that miR-29 regulates helper T cell differentiation by repressing multiple target genes, including at least two that are independently capable of inducing the Th1 gene expression program. Additional analyses of miRNA-mediated effects on other cytokines, including TNF, IL-4, and IL-13, revealed an ability of individual miRNAs to significantly influence the production of these cytokines as well. Notably, we found that miR-29 can promote TNF expression through effects that are dependent on the AU-rich element in the TNF 3'UTR. Taken together, these studies demonstrate the important role of individual miRNAs in cytokine regulation and raise intriguing possibilities for better understanding the gene expression networks that underlie immunity in health and disease.

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INTRODUCTION

Helper T cell differentiation and cytokine production

Coordinating and maintaining a healthy immune system involves the carefully orchestrated interaction of many different cell types and requires a complex network of cell-cell communication. In large part, this communication is mediated by the secretion of soluble factors known as cytokines. Cytokines signal through cell surface receptors and intracellular signaling pathways to influence the gene expression programs and thereby the functional activity of nearly all cells. While various cell types are capable of secreting cytokines and shaping the immune response, helper T cells represent a central source of these critical signaling molecules and disrupting cytokine production by these cells can have significant pathological consequences (1, 2).

Naïve $CD4^+$ helper T cells circulate through the blood and lymphoid organs surveying for antigen in a relatively quiescent and non-proliferative state. Upon antigen recognition by the T cell receptor, $CD4^+$ T cells integrate a complex set of signals from their environment. These signals include those from the T cell receptor itself, co-stimulation from antigen presenting cells, and input from the locally existing cytokine milieu. The result is helper T cell proliferation, migration,

and differentiation into effector subsets that are classified primarily by distinct cytokine production profiles. This ability to differentiate and secrete lineage specific cytokines is crucial to the effectiveness of helper T cells in the immune system as it allows for functionally different responses to different immune challenges. T helper 1 (Th1) cells secrete interferon-γ (IFN-γ) and Tumor necrosis factor-α (TNFα) and mediate elimination of intracellular pathogens. T helper 2 (Th2) cells are typically defined by their production of interleukin (IL)-4, IL-5, and IL-13 and play a central role in the elimination of extracellular parasites and helminthes. However, in the context of dysregulation and disease, Th1 cells and their cytokines can contribute to the inflammatory tissue damage of autoimmunity while Th2 cells and their cytokines are strongly associated with allergy and asthma.

Since the initial characterization of these two different helper T cell subsets over 25 years ago (3), at least two additional subsets have been described with unique cytokine expression profiles, gene expression programs, and contributions to both health and disease. IL-17 producing T helper 17 (Th17) cells have been demonstrated to direct the elimination of extracellular bacteria and fungi (4), yet can also play a significant role in many autoinflammatory pathologies (5). T regulatory (Treg) cells, on the other hand, represent a helper T cell lineage that functions to suppress the immune response, maintain immune tolerance, and prevent autoimmunity. Recent work has described even further specificity within the Treg lineage whereby Treg subsets can distinctively express chemokine receptors and/or cytokines of individual effector lineages as part of a

program to specifically regulate T cells of the corresponding effector subset (6-8). Additionally, there have been several reports of context-dependent situations in which helper T cells may produce cytokines of more than one defined subset (9). These findings raise the possibility that T cell differentiation involves at least some degree of plasticity and/or that individual T cells may exist along a multi-dimensional spectrum of the stereotyped lineages. Ultimately, these issues have significant implications for the development of inflammatory disease therapies that are aimed at inducing or reversing the differentiation of one T cell lineage in favor of another. Ongoing characterization of the signaling molecules, transcription factors, and epigenetic modifications involved in the initiation, reinforcement, and maintenance of T cell differentiation will continue to offer important insight into such therapies.

The details of T cell polarization involve several interacting pathways and many regulated changes in gene expression. Fundamental to this process, the complex set of environmental signals integrated during T cell activation influences the expression of lineage-specific transcription factors. Notably, this system is inherently subject to positive feedback loops whereby cytokine signaling can promote further production of the initiating cytokine and inhibit alternative cell fates. For Th1 cells, IFN-γ signaling through the IFN-γ receptor activates STAT1, which in turn can bind to the promoter of the T-bet gene (*Tbx21*) and activate its transcription (10). As the resulting T-bet protein can increase accessibility of the IFN-γ gene (11) and directly transactivate IFN-γ expression (12), a strong positive feedback loop for Th1 differentiation is readily

established. The positive feedback circuit of Th1 differentiation is further driven by effects of both T-bet and IFN-γ on the IL-12 pathway. IFN-γ induced T-bet drives expression of the IL-12 receptor on T cells (13) while IFN-γ also stimulates the production of IL-12 by antigen presenting cells (14). This increase in both IL-12 availability and IL-12 receptor expression leads to enhanced activation of the IL-12 responsive transcription factor, STAT4, and further augmented IFN-γ transcription during T cell receptor activation (13, 15). In this way, production of IFN-γ during the early phase of an immune reaction can establish a local environment that increasingly favors T-bet expression and Th1 differentiation in subsequently activated helper T cells. A similar positive feedback loop exists for Th2 cells whereby cytokine signaling through a STAT protein induces a lineage-specific transcription factor that is able to drive chromatin accessibility and ultimately increase transcription of the initiating cytokine. In the case of Th2 differentiation, IL-4 signals through STAT6 (16, 17) to induce GATA-3 and c-maf, resulting in additional IL-4 production and Th2 differentiation (18-20).

Due to the existence of these strong positive feedback loops in helper T cell differentiation, small changes in gene expression networks early in the differentiation process likely have significant consequences on the ensuing immune response. Along these lines, and underlying the research presented in this dissertation, microRNAs (miRNAs) have emerged in the last 10 years as important regulators of gene expression in nearly all cell types, including T cells and other lymphocytes. As “fine-tuners” of gene expression, these small RNA molecules are prime candidates for regulating the inherently sensitive feedback

loops of helper T cell differentiation and cytokine production. $CD4^+$ T cells that lack the machinery necessary to make mature miRNAs do indeed show defects in cytokine regulation, with a significantly increased propensity towards IFN-γ production (21, 22). Identifying and describing the regulation of cytokine production by specific miRNAs is the focus of the work presented here and remains an important objective in fully understanding the induction and maintenance of $CD4^+$ T cell fate decisions.

MicroRNA biogenesis and function

MicroRNAs represent an important class of evolutionarily conserved regulatory molecules involved in a wide range of cell functions and lineage decisions (23). Specifically, miRNAs are ~22 nt non-coding RNAs whose best known function is the post-transcriptional suppression of gene expression through either translational repression or mRNA destabilization and degradation (24, 25). A few reports also suggest that miRNAs can promote, rather than repress, the expression of certain targets in some situations (26, 27). Early findings in C. elegans suggested that the inhibitory, post-transcriptional effects of miRNAs on target genes were mediated primarily through translational repression, without large changes in transcript levels (28, 29). This notion persisted in the miRNA literature for several years, yet more recent genomic analyses of both transcripts and proteins suggest that miRNAs can in fact decrease mRNA abundance of target genes significantly (25, 30). The exact mechanism of miRNA mediated gene regulation remains an important area of

continued investigation and current work has not ruled out the possibility that the process may even differ among specific miRNA-target interactions.

The biogenesis of miRNAs (see Figure 1) typically begins with transcription of primary-miRNA (pri-miR) by Pol II in the nucleus (31). The miRNA itself may be encoded within an independent, non-protein-coding transcript or within the intronic region of a protein-coding transcript. Additionally, two or more miRNAs may be transcribed together as a "cluster" within one primary transcript, which is subsequently processed to release the individual miRNAs. Following transcription, the RNase III enzyme, Drosha, and its ds-RNA binding co-factor, DGCR8 (DiGeorge syndrome critical region gene 8), bind and process the primary transcripts into short hairpin precursor-miRNAs (pre-miRs) (32, 33). These ~70 nt products are transported into the cytoplasm where Dicer acts as both a ruler and a nuclease, removing the loop of the pre-miR to form a ~22 nt RNA duplex (34, 35). One strand of this duplex is then loaded into a protein-nucleic acid complex to form a miRNA-containing ribonucleoprotein complex (miRNP), also referred to as the RNA-induced silencing complex (RISC). This complex includes Argonaute among other proteins (36, 37) and is targeted to mRNA with sequence-mediated specificity. Notably, nucleotides 2-8 of the mature miRNA make up the 7 base pair "seed region" that is crucial for the sequence-dependent targeting of mRNA and is the basis of most target-prediction algorithms (38). miRNAs with similar seed regions can thus provide redundant effects on a given target while multiple miRNAs with different seed regions may cooperatively target one transcript. This simultaneously redundant

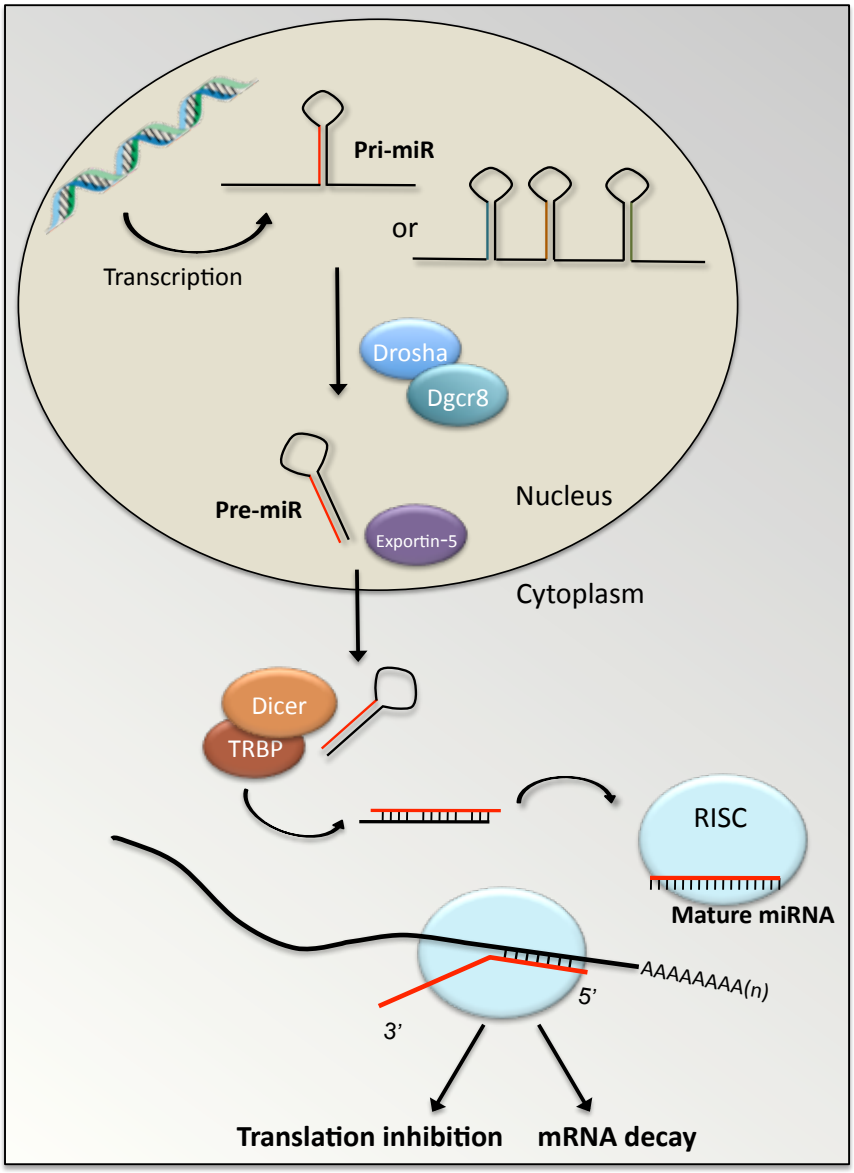

Figure 1. MicroRNA biogenesis and activity

Primary miRNA (pri-miR) transcripts are processed by Drosha and Dgcr8 to a precursor miRNA (pre-miR) hairpin that is exported to the cytoplasm where it is further processed by Dicer. One strand of the resulting double-stranded RNA is loaded into the RISC complex and directs repression of target mRNAs.

and cooperative nature of miRNA activity presents non-trivial challenges for determining functional contributions of individual miRNAs. Combining both gain of function and loss of function approaches, the research described here has utilized existing knowledge of miRNA biogenesis, mechanism of action, and target recognition in order to overcome some of these challenges and address the function of individual miRNAs in $CD4^+$ T cells.

MicroRNA function in $CD4^+$ T cells

Following the discovery and characterization of miRNAs, their expression and function in the immune system has become a topic of considerable interest. One of the first landmark studies to imply a function for miRNAs in immune cells involved the identification of specific miRNAs in mouse bone marrow. This study also described high expression of several miRNAs in hematopoietic and lymphoid tissues including the thymus (39). This early hint at the expression and importance of miRNAs in T cells was followed by additional miRNA profiling and functional studies that indeed established miRNAs as important regulators of T cell function. Notably, T cell-specific deletion of Dicer, and thereby miRNAs, resulted in significant developmental and functional defects. Dicer-deficient effector T cells exhibited decreased proliferation, increased apoptosis and dysregulated cytokine production (21, 40) while mice with specific deletion of Dicer from Treg cells developed severe autoimmunity (41, 42). These studies provide convincing evidence that miRNAs contribute significantly to the proper regulation of helper T cells, yet there are still only a limited number of instances

in which individual miRNAs and the molecular basis for their function have been identified in these cells.

Two reports that effectively demonstrate a functional role for a single miRNA in helper T cells involve work with miR-155 deficient mice (43, 44). These studies described marked defects in the germinal center response of miR-155 deficient mice and a significant Th2 differentiation bias in miR-155-deficient T cells (43, 44). The transcription factor c-maf was identified in these studies as a direct target of miR-155 that is at least partially responsible for the observed increase in IL-4 production by miR-155 deficient helper T cells. Subsequently, in one of very few studies to disrupt a miRNA binding site in vivo, knock-in mice containing a mutated miR-155 binding site in the 3'UTR of the *AICDA* gene were used to directly demonstrate the functional importance of a single miRNA-mRNA interaction (45). In regards to T cells, recent work suggests that miR-155 also regulates Treg homeostasis and survival (46) in addition to promoting inflammatory T cell development through effects on multiple cell types (47). Of specific relevance to the role of miRNAs in cytokine production, the proposed mechanism for regulation of Treg homeostasis by miR-155 involves direct targeting of SOCS1 (46), an established inhibitor of cytokine and STAT signaling. While the full complement of miR-155 function in T cells remains a topic of ongoing investigation, it is tempting to speculate about the possible effects that miR-155 and other miRNAs may have on T cell differentiation and cytokine signaling through regulation of SOCS1 and other SOCS proteins.

In addition to miR-155, miR-146a is one of the few individual miRNAs with a described function in helper T cells. Also like miR-155, a significant amount of the work on miR-146a involves its role in Treg homeostasis. MiR-146a deficient Tregs exhibit compromised regulatory function and increased effector cytokine production in cell transfer experiments and this effect appears to depend, in large part, on defects in STAT1 regulation (48). Despite the absence of a canonical, predicted miR-146a binding site in the STAT1 3'UTR, Lu et al. demonstrated a repressive effect of miR-146a on STAT1 expression in both Treg and T effector cells. MiR-146a-deficient $CD4^+$ effector cells had elevated STAT1 expression and increased IFN-γ production in vitro, suggesting this miRNA might also be in part responsible for the IFN-γ production bias of miRNA-deficient helper T cells. The details of miR-146a activity in effector cells may need further interrogation however, as independent analysis of miR-146a deficient T cells in vitro in our laboratory has not revealed the same effector cell differentiation defects. Finally, it worth noting that miR-146a was previously identified as one of very few miRNAs with significantly different expression in Th1 versus Th2 cells (49) and it remains the most consistently elevated miRNA in Th1 cells across additional studies (50). The increased expression of miR-146a in Th1 cells along with its ability to repress STAT1 suggests the possibility of an interesting negative feedback loop model whereby excessive IFN-γ signaling and Th1 differentiation could be kept in check by upregulation of miR-146a.

In addition to the few miRNAs that have been found to regulate transcription factors and cytokine signaling, miRNAs have also been identified

with other noteworthy functional effects in helper T cells. The miR-17~92 cluster comprises several miRNAs expressed as a polycistronic transcript and represents four miRNA seed families that are highly expressed in T cells. The high expression of this cluster has made it a popular focus for investigation. Xiao et al. demonstrated that transgenic mice overexpressing these miRNAs develop lymphoid hyperplasia and autoimmunity, with an especially noticeable increase in the proliferation and survival of $CD4^+$ T cells (51). This study and others indicate that the observed phenotype is the result of cooperative effects of multiple members of this miRNA cluster on the anti-apoptotic factor Bim, the tumor suppressor PTEN, and the cell-cycle regulator p21 (51-54). There has also been some success in dissecting the individual contributions of the several miRNAs within this cluster and the majority of the effects on cell proliferation and survival, especially in B cells, have been attributed to miR-17 and miR-19 (52, 55). Recent work has even suggested that miR-17 and miR-19 can also individually promote the differentiation of Th1 cells and IFN-γ production through effects on yet to be identified target genes (55). There is certainly much more to understand regarding the role of these highly expressed miRNAs in the expansion, differentiation, and effector function of helper T cells. This cluster of miRNAs, along with the closely related miR-106a and miR-106b clusters, remain an important example of the inherent challenges of studying individual miRNA function in the context of genomic clustering, multiple seed family members, and functionally overlapping target genes.

One of the first miRNAs to be cloned from mouse bone marrow, miR-181, also represents one of the few miRNAs with a well-described function in helper T cells. By negatively regulating several phosphatases involved in T cell signaling, miR-181 is able to increase the sensitivity of the T cell response to TCR stimulation (56). This is a particularly noteworthy example of the functional significance that can be achieved through the “fine-tuning” potential of miRNAs. Given the context of thymic T cell selection, even small changes in signal strength mediated by miR-181 can influence the T cell selection process, a fundamental aspect of immunity and tolerance. Indeed, by dampening TCR signals, miR-181 inhibition can promote the positive selection of self-reactive T cells that would otherwise be eliminated by negative selection (57). At the present time, the best-described functions of this miRNA involve its role in hematopoietic cell fate decisions and T cell signaling. Still, given the possible influence of signal strength on lineage differentiation (58), it remains an interesting possibility that miR-181 activity also affects T cell differentiation in certain settings. A deeper understanding of the coordinated regulation of miR-181 expression, T cell activation, and the effects of signal strength on T cell differentiation will offer further insight into this possibility.

Many individual miRNAs have been identified by microarray, Northern blot, quantitative RT-PCR, and deep sequencing that are highly expressed in $CD4^+$ T cells (39, 49, 50). Using these data as a starting point for loss of function and gain of function systems has led to the identification of miRNAs with effects on T cell proliferation, activation, and differentiation as described above. While

there are reports implicating a function for additional miRNAs in helper T cells, such as miR-326 in Th17 differentiation (59) and miR-182 in clonal expansion of effector T cells (60) the conclusions drawn from these studies stand to be strengthened by independent validation and the continued improvement of tools for in vivo targeting of miRNA activity. Nonetheless, there is little question that changing the expression of a single miRNA can have significant consequences on lymphocyte development and function, and in at least one case, on Th1 versus Th2 differentiation (43, 44). With this precedent established, there are still many miRNAs expressed in helper T cells for which little is known about the target genes and pathways under their regulation. The work presented in this dissertation is aimed at integrating miRNA biology into the field of helper T cell differentiation by 1) identifying miRNAs that underlie the severe defects of miRNA-deficient $CD4^+$ T cells and 2) describing the mechanisms of how these miRNAs normally function in immune regulation.

The microRNA-29 family

The microRNA-29 (miR-29) family is a highly conserved miRNA family that consists of three mature miRNAs in mice and humans. The human genomic organization of the miR-29 family involves a polycistronic gene encoding both miR-29b-1 and miR-29a on chromosome 7 and an additional cluster encoding miR-29b-2 and miR-29c on chromosome 1. The mature miRNA products of miR-29b-1 and miR-29b-2 are identical, but the pre-miRNAs and the corresponding antisense strand of these two genes are unique. In mice, the miR-29 genomic

organization is similar, with the miR-29b-1-29a cluster on chromosome 4 and miR-29b-2-29c on chromosome 13. Importantly, as miRNA family members, miR-29a, -29b, and -29c all share a common seed sequence and thus are likely to regulate a largely overlapping set of target genes.

Although best known for their regulation of target genes, the regulation of miRNAs themselves is also an important consideration in fully appreciating their function. To this extent, there is currently strong evidence to suggest that both the miR-29b-1/a and miR-29b-2/c clusters are subject to direct regulation by transcription factors. This includes transcriptional repression by c-myc and NF-κB and there is direct evidence for the latter in helper T cells in which NF-κB was shown to bind to a region immediately upstream of the miR-29b-1 hairpin sequence (61, 62). Additionally, although the sequence of mature miRNA-29 family members differ by only a few nucleotides, these differences may have important regulatory effects on both stability (63) and cellular localization (64). These findings suggest that there is much to learn about how subtle differences between miRNAs, and miR-29 family members in particular, may affect their expression and function in different cell types.

Profiling studies have observed significant expression of miR-29 family members in many cell types and several reports have described functions and disease associations of these miRNAs. A considerable portion of this literature has focused on an association of miR-29 with fibrosis (65-67) as well as a role in both acute myeloid leukemia (AML) (68) and chronic lymphocytic leukemia (CLL) (69, 70). Regulation of fibrosis by miR-29 appears to involve direct targeting of a

network of extracellular matrix proteins including collagens, fibrillins, and elastin (65). Regarding leukemia, miR-29 expression appeared lower in cancerous cells than normal cells and exogenous expression of miR-29 was able to induce apoptosis and decrease tumorigenicity. The tumor suppressor ability of miR-29 in these studies involved repression of MCL-1, CDK6, CXXC6 (TET1), DNMT3a, and DNMT3b in AML (68) and repression of the oncogenic TCL1 in aggressive CLL (69). However, the role of miR-29 as a tumor suppressor is likely complicated, for although miR-29 transgenic mice do not develop aggressive CLL, they do develop an indolent B-cell CLL (71).

Based primarily on predicted binding sites in the 3'UTR of both IFN-γ and T-bet, miR-29 represents an intriguing candidate for cytokine regulation. Prior to the work presented in this dissertation, however, miR-29 had not been shown to regulate immune function beyond the associations with cell survival and leukemia noted above. Concurrent with much of the work of this thesis, miR-29 has been independently demonstrated to repress IFN-γ production in vivo and also to regulate thymic homeostasis through effects on IFN-α receptor expression (62, 72). As more is discovered about the effects of this widely expressed miRNA family, interesting networks of gene interaction and cell function across multiple cell types are likely to emerge.

CHAPTER 2

MicroRNA-29 Regulates T-box Transcription Factors and Interferon-γ Production in Helper T Cells

David F. Steiner, Molly F. Thomas, Joyce K. Hu, Zhiyong Yang, Joshua E. Babiarz, Christopher D.C. Allen, Mehrdad Matloubian, Robert Blelloch, K. Mark Ansel

ABSTRACT

MicroRNA (miRNA)-deficient helper T cells exhibit abnormal IFN-γ production and decreased proliferation. However, the contributions of individual miRNAs to this phenotype remain poorly understood. We conducted a screen for miRNA function in primary T cells and identified individual miRNAs that significantly rescue the defects associated with miRNA deficiency. Multiple members of the miR-17 and miR-92 families enhanced miRNA-deficient T cell proliferation while miR-29 largely corrected their aberrant IFN-γ expression. Repression of IFN-γ production by miR-29 involved direct targeting of both T-bet and Eomes, two transcription factors known to induce IFN-γ production. Although not usually expressed at functionally relevant levels in helper T cells, Eomes was abundant in miRNA-deficient cells and was upregulated following miR-29 inhibition in wildtype cells. These results demonstrate that miR-29 regulates helper T cell differentiation by repressing multiple target genes, including at least two that are independently capable of inducing the Th1 gene expression program.

INTRODUCTION

$CD4^+$ helper T cells play a critical role in the coordination of effective immune responses. Upon activation, naïve $CD4^+$ T cells proliferate and differentiate into effector subsets defined primarily by distinct cytokine expression (73-75). As these cytokines act on many different cell types, the production and regulation of lineage specific cytokines is fundamental to generating the appropriate immune response for different types of immune challenges. Thus, proper regulation of helper T cell proliferation and differentiation is critical for effective immune protection from pathogens. However, dysregulated T cell responses can result in immunopathology. For example, Th1 cells secrete IFN-γ and mediate elimination of intracellular pathogens, but these cells can also contribute to pathologic inflammation and autoimmune disease. Examining the mechanisms of gene regulation that underlie T cell polarization has the potential to improve our understanding of cell differentiation in general and to provide insights for the development of clinically relevant immune therapies.

The differentiation fate of $CD4^+$ T cells involves integration of antigen, costimulatory, and cytokine signals that influence the expression level and duration of lineage-specific transcription factors. Enforced expression of T-bet dominantly induces IFN-γ production, and T-bet deficient $CD4^+$ T cells are severely defective in Th1 differentiation and IFN-γ production (12). Eomesodermin (Eomes), a closely related T-box family transcription factor, has also been shown to regulate IFN-γ production, particularly in $CD8^+$ T cells (76).

Although it is normally expressed at relatively low levels in $CD4^+$ T cells, Eomes can substitute for T-bet to induce IFN-γ production and Th1 differentiation when its expression is enforced. Once expressed, IFN-γ initiates a positive feedback loop that reinforces its own production and T-bet expression in helper T cells.

Recent work has identified endogenously expressed microRNAs (miRNAs) as important contributors to the regulation of helper T cell proliferation, survival, differentiation, and cytokine production (77). MiRNAs are ~22 nucleotide noncoding RNAs that mediate sequence-dependent post-transcriptional negative regulation of gene expression (78). Primary miRNA transcripts are processed by the Drosha-DGCR8 complex to yield ~60 to 80 nucleotide hairpin pre-miRNAs, which are subsequently cleaved by Dicer to form ~22 base pair dsRNA duplexes . One strand of this duplex forms the mature miRNA, which targets mRNAs for repression by complementary base pairing, especially within the “miRNA seed” sequence at nucleotide positions 2-8. Genetic inactivation of either Dicer or Drosha results in considerable functional defects in $CD4^+$ T cells (21, 22, 41, 42, 79). Dicer-deficient cells exhibit a marked bias towards IFN-γ production as well as significantly reduced proliferation and survival following stimulation in vitro. Similar phenotypes were observed in Drosha-deficient T cells (22). Although both Dicer and Drosha have been implicated in functions outside of miRNA biogenesis, the overlapping phenotypes of Drosha and Dicer deficient T cells indicate specific involvement of the miRNA pathway. These studies demonstrate the significance of miRNAs in regulating helper T cell gene expression, but the

individual miRNAs responsible and their mechanisms of function remain largely undefined.

Using T cells deficient for the essential miRNA processing co-factor, DGCR8, we established a system for analyzing the function of individual miRNAs in otherwise miRNA-deficient cells. Side-by-side comparisons showed that Dicer- and DGCR8-deficient helper T cells exhibit identical defects in cytokine regulation and proliferation. We further demonstrated that the phenotypes of miRNA-deficient cells result from cell-intrinsic defects, not feedback from aberrantly released cytokines, and that re-introduction of DGCR8 following stimulation can partially rescue the functional defects of these cells. Building on these findings, we carried out a functional screen and identified individual miRNAs that can alleviate the defects of miRNA-deficient cells.

RESULTS

DGCR8-deficient helper T cells

To establish a system for studying the functional effects of miRNAs in helper T cells, we intercrossed *Dgcr8*$^{fl/fl}$ mice (80) with mice expressing Cre under the regulation of the CD4 promoter/enhancer/silencer. *Dgcr8*$^{fl/fl}$ *CD4-Cre* mice were further crossed to *Rosa26-YFP* or *Rosa26-tdRFP* mice to introduce a fluorescent reporter of Cre expression (81). To confirm *Dgcr8* inactivation, we measured miRNA expression in the YFP^{+} population in cultured CD4^{+} cells from *Dgcr8*$^{fl/fl}$ *CD4-Cre Rosa26YFP* mice. DGCR8-dependent miRNA expression in

the *Dgcr8*$^{fl/fl}$ *CD4-Cre* YFP^{+} cells was below the limit of detection, while control cells expressed all miRNAs analyzed (Figure 1A). MiR-320 and miR-484 were still detected in *Dgcr8*$^{fl/fl}$ *CD4-Cre* YFP^{+} cells, confirming the DGCR8-independent expression of these two unusual miRNAs (82).

Analysis of lymph nodes and spleen revealed a decrease in the relative frequency of CD4 and CD8 cells by ~50% and ~66% respectively in *Dgcr8*$^{fl/fl}$ *CD4-Cre* mice (Figure S1A). This reduction is consistent with that reported for *CD4-Cre* mediated deletion of Dicer or Drosha (21, 22). We observed no significant difference in the frequency of naïve (CD62L^{hi}CD44^{lo}) cells among the CD4^{+} T cells in the lymph nodes and spleen of 4-7 week old *Dgcr8*$^{fl/fl}$ *CD4-Cre* mice (Figure S1B). This is likely due to the relatively young age of the mice used in this study as older mice with T cell specific deletion of miRNAs do accumulate previously activated helper T cells (22, 79) and data not shown). Moreover, counterselection against DGCR8-deficient T cells was not apparent in these mice, as no significant difference in the frequency of YFP^{+} cells among CD4^{+} T cells was observed between *Dgcr8*$^{fl/fl}$ *CD4-Cre* mice and *Dgcr8*$^{+/fl}$ *CD4-Cre* littermate controls (Figure S1C).

Like Dicer- and Drosha-deficient helper T cells, DGCR8-deficient T cells exhibited decreased proliferation (Figure 1B) and an overwhelming bias toward IFN-γ production in vitro (Figure 1C). The cytokine expression defect of DGCR8- and Dicer-deficient T cells was essentially identical, indicating that their common products, namely miRNAs, regulate helper T cell differentiation. Also consistent with previous reports (21, 22), cytokine production by wildtype and DGCR8-

deficient cells was similar in Th1 conditions and aberrant IFN-γ production was inhibited by Th2 culture conditions (Figure 1C-D). Further, anti-IFN-γ blocking antibody alone was sufficient to mediate this effect without the addition of IL-4 (Figure 1E). This result demonstrates that aberrant IFN-γ production in miRNA-deficient cells is dependent on IFN-γ signaling and specifically implicates genes in this pathway as potential targets of miRNA regulation.

Cell-intrinsic dysregulation of cytokine production in miRNA-deficient cells

Because IFN-γ induces a positive feedback loop through STAT1 and T-bet to reinforce its own expression (10, 13), we tested the possibility that an increase in early IFN-γ release could be the proximal cause of the IFN-γ production bias observed in DGCR8-deficient T cell cultures. *Dgcr8*$^{fl/fl}$ *CD4-Cre Rosa26-tdRFP* cells were co-cultured with *Dgcr8*$^{+/fl}$ *CD4-Cre Rosa26-YFP* control cells. This system allowed us to distinguish the miRNA-deficient RFP^+ cells from the control YFP^+ cells at the time of analysis (Figure 2A). DGCR8-deficient T cells retained their Th1 bias and IFN-γ production in co-culture. However, despite exposure to the same cytokine environment as the miRNA-deficient cells, control cells did not exhibit increased IFN-γ production. To better understand this cell-intrinsic difference in cytokine production we analyzed additional aspects of the IFN-γ feedback loop. Activated DGCR8-deficient cells exhibited higher levels of both phosphorylated STAT1 (pSTAT1) and T-bet relative to co-cultured control cells (Figure 2B). There is evidence that the gene encoding the IFN-γRα subunit (*Ifngr1)* can be regulated by at least one miRNA (83), but we did not observe

increased surface expression of this receptor on unstimulated miRNA-deficient cells (Figure 2C). Additionally, naïve wildtype and miRNA-deficient cells expressed the same basal levels of T-bet (Figure 2C) and negligible amounts of pSTAT1 (Figure 2D, *shaded histograms*). Notably however, following treatment with recombinant IFN-γ, naïve DGCR8-deficient T cells exhibited higher levels of STAT1 phosphorylation than control cells (Figure 2D, *open histograms*). We conclude that cell-intrinsic defects sensitize the IFN-γ signaling pathway in miRNA-deficient T cells, thus leading to aberrant Th1 differentiation and IFN-γ production.

To further examine the requirements for the miRNA pathway in the cell-intrinsic control of IFN-γ production, we used retroviral transduction to re-express DGCR8 in differentiating DGCR8-deficient T cells. Those cells that were successfully transduced with the *Dgcr8*-containing retrovirus exhibited improved proliferation and a significant decrease in the frequency of IFN-γ producing cells (Figure S2A and data not shown). However, untransduced cells in the same culture remained significantly biased towards IFN-γ production. Cytokine production by miRNA-deficient T cells was also not affected by a control retrovirus containing only the Thy1.1 marker gene (Figure S2A). DGCR8 expression led to the expected re-expression of mature miRNAs (Figure S2B). Taken together, these results indicate that miRNAs dynamically regulate cytokine production in helper T cells in a cell-autonomous manner, and that miRNA activity has significant effects on IFN-γ production even after initial activation and polarization events have occurred.

A screen for individual miRNA functions in CD4$^+$ T cells

After observing the effect of re-introducing DGCR8, we reasoned that some subset of the miRNAs expressed in T cells must be responsible for the phenotypic rescue. In order to identify the miRNAs involved, we implemented a system to screen for individual miRNA functions in differentiating CD4$^+$ T cells. Due to the often cooperative and redundant nature of miRNA function, there are inherent difficulties in studying individual miRNAs. To overcome some of these difficulties we utilized *Dgcr8$^{fl/fl}$ CD4-Cre* mice to allow introduction of individual miRNAs into otherwise miRNA deficient helper T cells, an approach that proved to be a useful and sensitive means for identifying the function of individual miRNAs in embryonic stem cells (84).

With the goal of delivering individual miRNAs to DGCR8-deficient T cells, we first optimized the transfection of primary CD4$^+$ cells by electroporation (85). Using siRNA to knock down YFP expression in *Dgcr8$^{fl/fl}$ CD4-Cre* YFP$^+$ cells, we were able to achieve transfection efficiency of greater than 90% with minimal effects on cell viability (data not shown). To observe changes in miRNA activity, we generated retroviral sensor constructs containing four miRNA binding sites downstream of a GFP coding region. As expected, sensor GFP expression was significantly repressed in miRNA-expressing wildtype cells as compared to DGCR8-deficient cells (Figure S3A). We next used these sensors to validate miRNA gain of function following transfection of primary T cells with synthetic miRNA oligonucleotides. In DGCR8-deficient cells, GFP expression from a miR-

29a sensor was repressed following transfection with miR-29a, but not miR-150 (Figure S3B). Similarly, a sensor with miR-150 binding sites was repressed by miR-150 but not miR-29a (Figure S3B). These results indicate that synthetic miRNA oligonucleotides can be successfully introduced into primary T cells with high efficiency and exhibit sequence-specific repression of target genes, even in T cells that are otherwise deficient in their ability to generate mature miRNAs.

We next sought to determine the appropriate set of miRNAs for analysis in a functional screen. Small RNA libraries for deep sequencing were generated from wildtype $CD4^+$ T cells activated *in vitro* for 44 hours, providing a relevant "snapshot" of the miRNAs present near the time of miRNA introduction in our screen (Figure 3A, Table S1). From these data we chose 110 miRNAs to screen for function in DGCR8-deficient helper T cells. All miRNAs for which we obtained at least 100 reads per million total reads from two independent sequencing libraries were included.

A schematic of the screening procedure is depicted in Figure 3B. CFSE-labeled DGCR8-deficient T cells were activated *in vitro*, transfected with individual miRNAs after 24 hours, returned to stimulus for an additional ~40 hours, and then cultured in media containing IL-2. Proliferation was measured by CFSE dilution on the fourth day, and the remaining cells were transfected again prior to cytokine analysis to overcome miRNA turnover and dilution. On the next day, cells were restimulated and stained intracellularly for flow cytometric analysis of cytokine production.

miR-17 and miR-92 family miRNAs promote T cell proliferation

An initial screen of our T cell miRNA library revealed several miRNAs with a positive effect on proliferation (Figure 3C and Table S2). Of the 10 miRNAs that most significantly increased proliferation, five (miR-17, miR-20a, miR-93, miR-106a, miR-106b) belong to the miR-17 seed family and an additional three (miR-25, miR-32, miR-92a) share the miR-92 seed sequence. This represents three of four miR-25 seed family miRNAs assayed and five of six miR-17 family miRNAs assayed (Figure 3D). These findings are consistent with the established role for the miR-17~92 cluster in regulating proliferation of multiple cell types (86), including its ability to induce lymphoproliferative disease when overexpressed in mice (51, 87). The other two miRNA seed families that are represented in the miR-17~92 cluster, miR-18 and miR-19, did not promote proliferation in our screen.

To validate these results, we repeated proliferation assays following transfection with individual members of the miR-17~92 cluster (Figure 3E). These experiments verified that miR-17 and miR-92a both increase the proliferation index of DGCR8-deficient T cells approximately 10%, representing a partial but consistent rescue ($p<0.01$ ANOVA, Tukey's post hoc test). To address the mechanism of this effect, we analyzed the expression of validated miR-17~92 targets with established roles in cell proliferation and survival. Previous reports have identified Pten as a target of miR-17 and miR-19 (51, 52), Bim as a target of miR-17 and miR-92 (51-54), and p21 as a target of the miR-17 seed family (54, 84, 88). However, these targets were not significantly affected by transfection

with miR-17 or miR-92a, suggesting that additional targets may be responsible for the pro-proliferative effects of these miRNAs in helper T cells (Figure S4A). In addition, miR-17 and miR-92a increased proliferation without observable effects on cell survival, whereas miR-19 increased cell viability relative to control transfected cells but did not augment proliferation (Figure S4B). Taken together, these results demonstrate the effectiveness of this screening approach for identifying specific functional capabilities of individual miRNAs.

miR-29a and miR-29b regulate IFN-γ production

Building on our success in identifying miRNA functions that are consistent with their known biological activities, we next screened for individual miRNAs that can rescue the aberrant IFN-γ production of DGCR8-deficient T cells. In this screen, both miR-29a and miR-29b significantly decreased the frequency of IFN-γ producing cells (Figure 4A and Table S3). The only other member of this seed family, miR-29c, was not included in our screen due to its low expression in helper T cells (Figure S5). Additional transfection experiments confirmed the ability of both miR-29a and miR-29b to reduce IFN-γ production in DGCR8-deficient T cells (Figure 4B) and that this effect was dose-dependent (Figure 4C). MiR-29 had no significant effect on the total frequency of IL-4 or IL-2 producing cells (Figure 4D), cell proliferation (Figure 4E), or viability (Figure 4F). MiR-29 also repressed IFN-γ production in wildtype cells, even in conditions that strongly promote Th1 differentiation (Figure 4G-H). Taken together, these findings indicate that miR-29 mediates its effects through specific regulation of the IFN-γ

production pathway rather than through general effects on T cell activation, cytokine production, or cell fitness.

Genome-wide analysis identifies T-bet and Eomes as direct targets of miR-29

To identify miR-29 targets that contribute to IFN-γ regulation, we conducted microarray analyses of gene expression in both miR-29 gain and loss of function conditions. A microarray-based approach to target identification is supported by recent evidence that miRNA-mediated changes in mRNA and protein levels are highly correlated (25) For gain of function experiments we transfected DGCR8-deficient cells with synthetic miR-29b and for loss of function experiments we transfected wildtype cells with combined miR-29a and miR-29b antisense inhibitors. As a class, predicted miR-29 targets (89) were clearly repressed following miR-29b transfection in DGCR8-deficient cells (Figure 5A, left panel). Of the 115 predicted miR-29 targets whose expression was significantly changed (FDR<.1), 111 were downregulated and only 4 exhibited increased expression (Figure 5B and Table S4). Predicted targets were also upregulated as a class following inhibition of miR-29 in wildtype T cells (Figure 5A, right panel). Notably, the majority of predicted miR-29 targets that were downregulated in response to miR-29b in DGCR8-deficient cells were also upregulated in wildtype cells transfected with miR-29 inhibitors (Figure 5B). Taken together, these data indicate that many of these genes are not only capable of responding to miR-29 overexpression, but are in fact subject to

regulation by endogenous miR-29 in wildtype T cells. Among the genes regulated in this manner, we identified several previously validated targets of miR-29 including Dnmt3a, Dnmt3b, Cdk6, and Mcl1 (90-92) (Figure 5C). Many genes identified in this analysis likely contribute to the overall function of miR-29 in helper T cells.

In regards to the regulation of IFN-γ specifically, we were intrigued that both *Tbx21* (encoding T-bet) and *Eomes* appeared to be regulated by miR-29. Both T-bet and Eomes have known roles in IFN-γ production and both contain highly conserved 3'UTR miR-29 binding sites. We validated the microarray findings for these genes by qPCR and found a 50% reduction of *Tbx21* mRNA following miR-29 transfection in miRNA-deficient cells and a 30% increase following miR-29 inhibition in wildtype cells (Figure 5D). Eomes mRNA levels were affected to an even greater extent, with expression reduced by 80% in miR-29-transfected DGCR8-deficient cells and increased by 70% following miR-29 inhibition in wildtype cells (Figure 5D).

To determine whether T-bet and Eomes are in fact direct targets of miR-29, we transfected primary T cells with dual luciferase reporters containing the full length mouse *Tbx21* or *Eomes* 3'UTR. In DGCR8-deficient T cells, miR-29b reduced expression of the co-transfected *Tbx21* 3'UTR reporter by approximately 65% and miR-29b reduced expression of the Eomes 3'UTR reporter by 80% (Figure 5E). In wildtype T cells, miR-29 inhibition significantly "de-repressed" the full length *Tbx21* 3'UTR and *Eomes* 3'UTR reporters by 20% and 40%, respectively (Figure 5F). These results demonstrate that both the *Tbx21* and

Eomes 3'UTRs are directly responsive to miR-29, and that physiological levels of miR-29 in wildtype helper T cells are sufficient to mediate these effects.

miR-29 regulates T-box transcription factor protein expression in vitro and in vivo

Intracellular staining confirmed that miR-29 returned T-bet protein expression in miRNA-deficient cells to a level near that of wildtype cells (Figure 6A). Although Eomes is typically expressed only at low levels in $CD4^+$ T cells, its expression was also significantly elevated in miRNA-deficient $CD4^+$ cells and miR-29 transfection rescued this aberrant expression (Figure 6B).

To analyze miR-29 function in helper T cells in vivo, we retrovirally transduced SMARTA $CD4^+$ TCR transgenic T cells with either pri-miR-29a or control pri-miR-1. Transduced cells were adoptively transferred and recipient mice were infected with LCMV one day later. On day 8 post transfer, we analyzed T-bet and Eomes expression in transduced cells. As expected of wildtype $CD4^+$ cells, Eomes expression was negligible regardless of miR-29 overexpression (data not shown). However, T-bet expression was significantly reduced in the miR-29 transduced cells (Figure 6C). We also observed significantly decreased IFN-γ production by miR-29 overexpressing cells, as indicated by decreased MFI among $CD4^+$ effector cells producing both IFN-γ and TNF-α (Figure 6D).

To further assess the in vivo role of T-bet regulation by miR-29, we conducted additional experiments with p14 LCMV-specific TCR transgenic $CD8^+$

T cells. Again we observed a decrease in T-bet in miR-29 overexpressing cells (Figure 6E). Furthermore, we observed fewer KLRG1$^+$ IL-7Rα$^-$ cells and an increased frequency of KLRG1$^-$ IL-7Rα$^+$ cells (Figure 6F). Despite the relatively modest reduction observed in T-bet in CD8$^+$ cells, these results are consistent with reports that development of KLRG1$^+$ IL-7Rα$^-$ short lived effector cells (SLEC) is particularly sensitive to graded expression of T-bet (93). Taken together, these results demonstrate the ability of miR-29 to functionally contribute to T-bet regulation in both CD4$^+$ and CD8$^+$ T cells in vivo.

Regulation of IFN-γ by miR-29 involves both T-bet and Eomes

To measure the functional relevance of T-bet and Eomes as miR-29 targets, we determined if direct T-bet and Eomes knockdown with siRNA would phenocopy miR-29 gain of function in DGCR8-deficient T cells. T-bet siRNA reduced T-bet expression below the level observed in miR-29 transfected cells (Figure 7A) and did lead to a significant reduction in IFN-γ production. (Figure 7C). Surprisingly however, T-bet knockdown was not sufficient to reduce IFN-γ production to the same extent achieved by miR-29b (Figure 7C). Direct knockdown of Eomes alone had no effect on IFN-γ production (Figure 7B-C), likely due to a dominant effect of T-bet on IFN-γ production in these cells. However, combined knockdown of both T-bet and Eomes reduced IFN-γ production to a greater extent than independent knockdown of T-bet alone (Figure 7C). Thus, T-bet expression does influence IFN-γ production and miR-29 does repress T-bet, but T-bet repression alone cannot fully account for the ability

of miR-29 to regulate IFN-γ. While we must acknowledge that additional miR-29 targets may also contribute, we conclude that miR-29 regulates IFN-γ production by simultaneously targeting T-bet and Eomes, two proteins with overlapping transcriptional activity.

DISCUSSION

We and others have previously shown that miRNA deficient helper T cells exhibit significant defects in proliferation and the regulation of cytokine production. However, defining the contributions of specific miRNAs to these processes has been challenging due to cooperation and redundancy among miRNAs. In this study, we utilized T cells from *Dgcr8*$^{fl/fl}$ *CD4-Cre* mice as a source of miRNA-deficient cells into which individual miRNAs could be reintroduced and assayed for their ability to rescue phenotypes associated with miRNA deficiency. Optimization of primary T cell transfection allowed an efficient functional screen of more than 100 miRNAs in primary T cells. Starting with this screen, we identified individual miRNAs that rescue the defects of miRNA-deficient T cells and further determined the molecular mechanism of this activity. MiR-17 and miR-92 family members supported proliferation of DGCR8-deficient T cells, whereas miR-29 family members potently and specifically inhibited IFN-γ production. MiR-29 directly targeted T-bet and its close relative Eomes. Both endogenous and overexpressed miR-29 inhibited the expression of these key transcriptional regulators of IFN-γ expression. This coordinated repression of

functional homologs by a single miRNA represents a novel mode of cell fate regulation, and may apply broadly to miRNA function in other settings.

Introduction of miRNAs into otherwise miRNA-deficient cells proved to be an especially sensitive system for detecting bona fide functional effects. Transfection of wildtype cells with miR-29 had relatively modest effects on IFN-γ production (data not shown), suggesting that it may already be functioning near saturating levels. Indeed, the miR-29, -17, and -92 seed family miRNAs are among the most highly expressed miRNAs in activated $CD4^+$ T cells. We also considered the possibility that a screen of all known miRNAs could reveal "artificial" functions for miRNAs not normally expressed at meaningful levels. For this reason, we limited our screen to miRNAs expressed in activated $CD4^+$ T cells. Further analysis of the regulation of miRNA expression during T cell differentiation may reveal optimal conditions for modulating miRNA activity and analyzing functional effects and target genes in wildtype T cells.

Multiple members of the miR-17 and miR-92 miRNA families were able to promote proliferation of DGCR8-deficient helper T cells. All three clusters containing these miRNAs are expressed in helper T cells, and two of them (miR-17~92 and miR-106a~363) are strongly induced upon T cell activation (50, 94). Overexpression of the entire miR-17~92 cluster promotes lymphomagenesis in B cells and can substitute for co-stimulation to promote helper T cell proliferation (51, 87). Our screening system allowed us to dissect the functional contribution of individual miRNAs within these clusters. MiR-17 and miR-92 family members were key players in regulating the rapid proliferation of activated T cells. Of note,

miRNAs with the miR-17 seed sequence also promote cell cycle progression in embryonic stem cells and human cell lines (54, 84, 88). Clarifying the regulatory networks that enable miR-17~92 miRNAs to cooperatively regulate cell proliferation and survival will be important to fully understand their role in immunity and immune dysfunction.

MiRNA effects on cell proliferation and survival appear to be nearly universal in diverse somatic cell types. In contrast, the increased propensity of both Dicer- and DGCR8-deficient helper T cells to produce IFN-γ reveals a noteworthy case of a cell-type specific immune function that is regulated by the miRNA pathway. Our findings demonstrate that miR-29 is an important regulator of the IFN-γ pathway in helper T cells, and that this regulation is mediated in part through the Th1 transcription factor T-bet. T-bet heterozygous mice, which exhibit only a 50% reduction in T-bet protein, spontaneously develop Th2 airway inflammation (95). Thus, even moderate repression of T-bet by miR-29 may have important physiological implications. Consistent with the well-established role of T-bet in promoting IFN-γ production, siRNA-mediated reduction of T-bet did repress IFN-γ in our experiments. Surprisingly however, miR-29 had a far more potent effect than T-bet knockdown alone. These findings indicate that the effects of miR-29 on IFN-γ are mediated through both T-bet and additional targets and support the notion that miRNAs rarely function through repression of a single target gene.

Microarray analysis following miR-29 gain of function and loss of function demonstrated that many genes with canonical miR-29 binding sites are

significantly repressed by both overexpressed and endogenous miR-29. These data confirm previously validated targets of miR-29 and identify many novel candidate targets. At least one previously validated target, Dnmt3a, has been implicated in the regulation of cytokine production (96), and may also be involved in the observed function of miR-29 reported here. Of particular interest to this study was *Eomes*, which encodes a T-box transcription factor closely related to T-bet. While Eomes is known to be important for IFN-γ production in $CD8^+$ T cells, it is normally expressed at very low levels in $CD4^+$ effector cells. However, ectopic expression of Eomes can promote IFN-γ production in $CD4^+$ cells and mechanisms for the transcriptional repression of Eomes in $CD4^+$ cells have been described (76, 97, 98). These findings underscore the importance of maintaining *Eomes* silencing for proper control of helper T cell differentiation and cytokine production. Conceivably, even transient or minimal expression of Eomes could initiate the IFN-γ/T-bet positive feedback loop that drives Th1 differentiation. We found that miRNA-deficient $CD4^+$ cells did not appropriately restrict T-bet or Eomes, and RNAi experiments showed that both contributed to aberrant IFN-γ production. The direct targeting of both T-bet and Eomes by miR-29 implicates a central role for this miRNA in the phenotype of miRNA-deficient $CD4^+$ T cells. However, additional miRNAs likely contribute as well, perhaps through effects on transcription factors such as GATA3 and Runx3 that can also regulate Eomes and IFN-γ expression (97). *Ifng* itself has also been described as a computationally predicted and functionally relevant target of miR-29 (99) and Smith et al., co-submitted manuscript). However, we could not confirm any effect

of miR-29 on a full-length *Ifng* 3'UTR reporter, perhaps due to the dominant miRNA-independent regulation of the *Ifng* 3'UTR in helper T cells (100).

Taken together, our data suggest that miR-29 may have evolved as an important regulator of $CD4^+$ effector T cell cytokine production, both by modulating the level of the lineage-specific transcription factor T-bet and by restricting leaky transcription of the functionally overlapping T-box transcription factor Eomes. $CD4^+$ T cells are distinguished by their ability to undergo functional polarization into diverse effector subsets, some of which depend on silencing of *Ifng* for proper immune function. Therefore, $CD4^+$ T cells must have mechanisms available for robust regulation of both T-bet and Eomes. In contrast, miRNA-deficient NK cells and $CD8^+$ T cells do not exhibit increased IFN-γ production as $CD4^+$ cells do (101, 102). However, since activated NK and CD8+ T cells express T-bet and Eomes, miR-29 likely regulates their expression in these cells as well. Indeed, we observed decreased T-bet in $CD8^+$ T cells overexpressing miR-29, and this corresponded with a significant decrease in the T-bet-dependent SLEC population during an in vivo immune response to LCMV.

Current models of helper T cell differentiation emphasize the role of cytokine and transcription factor positive feedback loops that polarize gene expression patterns. This feature makes T cell fate decisions very sensitive to small changes in the expression of key genes in the regulatory circuit, and therefore especially amenable to regulation by miRNAs. Indeed, multiple molecular players in the Th1 pathway have now been characterized as targets of miRNAs. In addition to the current findings regarding miR-29, miR-155 and miR-

146 have been shown to target *Ifngr1* and *Stat1*, respectively, and miR-155-deficient T cells display dysregulated Th2 differentiation (43, 44, 48, 83). We did not observe increased IFNGR1 expression in DGCR8-deficient T cells, and miR-155 was excluded from our primary screen because it also compromised T cell survival. STAT1 was increased in DGCR8-deficient cells, and miR-146 transfection 24 hours after T cell activation consistently but modestly repressed IFN-γ production. It is possible that even earlier introduction of miR-146 would have had a larger effect on IFN-γ production. To this extent, naïve DGCR8-deficient T cells exhibited increased phosphorylated STAT1 in response to IFN-γ as compared to wildtype cells. This indicates a role for miRNA regulation of IFN-γ signaling prior to the time when miRNAs were reintroduced into T cells in this study. Thus, our screen may underestimate the role of miR-146 and other miRNAs whose targets may be most critical for very early events in T cell activation and differentiation.

In conclusion, we have identified individual miRNAs that have significant effects on proliferation and cytokine production by helper T cells. These processes are central to appropriate immune system function and their dysregulation can have significant pathological consequences. As such, miR-29 regulation of IFN-γ production and helper T cell differentiation has important implications for human diseases that are associated with T cell-mediated immunity such as asthma, type 1 diabetes, and multiple sclerosis (MS). Indeed, recent findings suggest that low expression of miR-29 in the T cells of MS patients contributes to the T-bet- and IFN-γ-mediated inflammation associated

with this disease (Smith et al., co-submitted manuscript). Understanding the complete set of functions for miR-29 and other miRNAs in helper T cells will likely provide useful insights for the development of novel immune therapies. Such therapies may focus on the miRNAs themselves or may modulate pathways implicated through characterization of miRNA target genes. Our results broaden our understanding of genome regulation by miR-29 and may have implications for other biological and disease processes that have also been linked to miR-29 function including fibrosis, HIV latency, and leukemia (65, 103, 104).

EXPERIMENTAL PROCEDURES

Mice

Dgcr8$^{fl/fl}$ and *Rosa26-Stop-loxp-tdRFP* have been described previously (80, 81). C57BL/6 (NCI), *CD4-Cre (*Taconic), *Rosa-26-Stop-loxp-YFP,* SMARTA, and P14 mice (Jax) were purchased. All mice were housed and bred in specific pathogen-free conditions in the Animal Barrier Facility at the University of California, San Francisco. All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of California, San Francisco.

T cell stimulation and transfection

$CD4^+$ T cells from spleen and lymph nodes of young mice (4-7 weeks old) were isolated by magnetic bead selection (Dynal), stimulated with anti-CD3/28, and cultured in polarizing conditions as indicated. Cells were transfected using the

Neon electroporation transfection system (Invitrogen) with an optimized version of the manufacturers recommended protocol. miRIDIAN miRNA mimics, inhibitors, controls, and T-bet siRNA were from Dharmacon. Eomes siRNA was from Qiagen. (See supplement for details).

miRNA screening and analysis

Cells were stimulated at 1 x 10^6 cells/mL for 24 hour in 6-well plates. Cells were removed from stimulation, pooled, and 0.2 x 10^6 cells were transfected with an individual miRNA. Following transfection, cells were placed in goat anti-hamster IgG-coated wells of a 24-well plate in 0.5 mL media with anti-CD3/28. Availability of mice and cells allowed 16-24 miRNAs to be screened at one time such that the complete screen of all miRNAs consisted of 5 batches. CFSE analysis on day 4 was used to calculate a proliferation index. Proliferation index is the average number of cell divisions among cells that underwent at least one division (Flowjo analysis software). 0.2 x 10^6 cells of the remaining cells from each well were transfected again on day 4 and restimulated for intracellular staining on day 5. For each batch, the proliferation index and the frequency of IFN-γ producing cells was normalized to the batch median to give a proliferation score and IFN-γ score, respectively. These values were then used to generate *Z* scores for the entire set of miRNAs screened (*Z* = *x*-mean/SD, where *x* is the proliferation score or IFN-γ score for each individual miRNA).

Adoptive Transfer and LCMV infection

$CD4^+$ T cells from SMARTA mice (CD45.1) were isolated by magnetic bead selection, activated in vitro as described, and transduced with pri-miR retroviral vectors (pri-miR-29a or pri-miR-1) at 40 h. At 45 hours cells were washed, resuspended in PBS, and 5 x 10^4 cells were injected i.v. into recipient CD45.2 C57BL/6 mice (NCI). One day later mice were infected i.p. with 2 x 10^5 plaque forming units of LCMV. On d8 post-transfer, total splenocytes were harvested and immediately fixed for analysis. $CD8^+$ cells were activated *in vivo*, transduced, and transferred as described (105) and splenocytes were analyzed on d7 post-transfer.

Microarray procedures and analysis

Sample preparation, labeling, array hybridizations, and false discovery rate calculations were performed according to standard protocols from the UCSF Shared Microarray Core Facilities and Agilent Technologies (http://www.arrays.ucsf.edu and http://www.agilent.com) as previously described (106).

Supplemental Experimental Procedures

T cell stimulation and culture

Purified $CD4^+$ T cells were stimulated with hamster anti-mouse CD3 (clone 2C11, 0.25 µg/mL) and anti-mouse CD28 (clone 37.51, 1 µg/mL) on plates coated with goat anti-hamster IgG (0.3 mg/mL in PBS for 2 hours at room temperature; MP Biomedicals) for 60-68 hours at an initial cell density of 0.7-1 x 10^6 cells/mL.

Following stimulation, cells were expanded in media with 20 units/mL of recombinant IL-2 (National Cancer Institute). ThN (non-polarizing, no exogenous cytokines or blocking antibodies), Limiting IL-4 (10units/mL IL-4), anti-IFN-γ only (5 μg/mL anti-IFN-γ), Th2 (500 units/mL IL-4, 5 μg/mL anti-IFN-γ clone XMG1.2), or Th1 (10ng/mL IL-12, 10 μg/mL anti-IL-4) conditions were maintained throughout stimulation and expansion. The resulting cultures were free of $CD8^+$ T cells (<2%) when analyzed by flow cytometry 5 d after activation. For experiments involving carboxyfluorescein succinimidy ester (CFSE), cells were labeled for eight minutes with 5 uM CFSE, quenched with an equal volume of fetal bovine serum (FBS), and washed twice in 10% FBS prior to stimulation and culture. T cell culture was in DMEM high glucose media supplemented with 10% FBS, pyruvate, nonessential amino acids, MEM vitamins, L-arginine, L-asparagine, L-glutamine, folic acid, beta mercaptoethanol, penicillin, and streptomycin.

T cell transfection and synthetic miRNA and siRNA oligonucleotides.
Transfections were performed using 2-3 x 10^7 cells/mL in 10 μL "R buffer" (Invitrogen) with a RNA concentration of 500 nM unless otherwise specified. Optimized setting used was 1550 V with three 10 ms pulses. All transfection experiments involving cytokine analysis consisted of one transfection after 24 h of stimulation and a second transfection at 90-100 h. For the initial transfection, cells were removed from plates, transfected, and returned to plate-bound

stimulation (anti-CD3/28). Following the second transfection, cells were returned to media with 20 units/mL IL-2.

Mouse Tbx21 siRNA #1; target sequence: ACACACACGUCUUUACUU).

Mouse Eomes siRNA #3); target sequence: AACACTGAAGAGTACAGTAAA).

miR-29a and miR-29b antisense hairpin inhibitors (Dharmacon) were combined and used at 500 nM of each. Negative control miRNA and inhibitor were based on cel-miR-67 (mature sequence UCACAACCUCCUAGAAAGAGUAGA, Dharmacon).

Dgcr8, pri-miR constructs, and miRNA sensors

Dgcr8 cDNA was subcloned into MSCV-IRES-Thy1.1 DEST (www.addgene.org: plasmid 17442). Pri-miR constructs were generated by amplifying and subcloning the endogenous miRNA hairpin with ~150 nucleotides of 5' and 3' flanking sequence into MSCV-IRES-Thy1.1 DEST. Pri-miR-29a parent construct was provided by A.Goga. MiRNA sensor retroviral constructs were generated by subcloning 4 perfectly complementary miRNA binding sites immediately downstream of a GFP coding gene with each miRNA binding site separated by 4 base pairs. The GFP-miRNA binding site (GFP-miR-BS) segment was then inserted into a MSCV-PGK-hCD25 retroviral construct to provide expression of a human CD25 reporter gene that is not affected by miRNA activity. Cells transduced with MSCV-GFP-miR-BS-PGK-hCD25 retroviruses were transfected with synthetic miRNA oligonucleotides on day 4, and analyzed for GFP expression on day 5. To control for possible differences in transduction efficiency

between samples, GFP analysis was limited to cells with matched surface expression of the miRNA-independent hCD25 transduction reporter gene. For luciferase reporter assays, the full-length 3'UTR of *Tbx21* and *Eomes* were amplified from murine activated T cell cDNA with the following primers and cloned into psiCHECK-2 (Promega):

T-bet fwd, 5'-CTCGAGGAAAATGCCGCTGAATTG-3'

T-bet rev, 5'-GCGGCCGCTTTACCAGGTCCATGTTTATTTC-3';

Eomes fwd, 5'-CTCGAGAGCATTATTTTAACCTTTAACC-3';

Eomes rev, 5'-GCGGCCGCTACAGAAGACAGAGCTATACC-3'.

Retroviral Transduction

$CD4^+$ cells were stimulated as described for 48 hours and transduced with retrovirus produced by Phoenix-E packaging cells transfected with retroviral plasmids. Following 6 hours of incubation with virus and 8 μg/mL polybrene, media was replaced and cells were cultured and expanded for analysis.

Intracellular staining and antibodies

After 5 days of culture, cells were restimulated for 4 hours with 10 nM phorbol 12-myristate 13-acetate (PMA) and 1 μM ionomycin in the presence of 5 μg/mL brefeldin A, fixed with 4% formaldahyde and permeabilized and stained in PBS containing 0.5% saponin, 1% bovine serum albumin (BSA), and 0.1% sodium azide. For phosphorylated (Y701) STAT1, cells were fixed and permeabilized in 100% methanol and stained in PBS containing 1% BSA. Fluorophore-conjugated

antibodies for Eomes, T-bet, IFN-γ, and IL-4 were from eBioscience. APC-pSTAT1 and biotin-IFNGR1 were from BD Biosciences. Data was collected using a LSRII with FACSDiva software (BD Biosciences), and analyzed with Flowjo software.

RNA isolation and Quantitative PCR

Total RNA was isolated with Trizol LS reagent (Invitrogen). For mRNA expression analysis cells were harvested on day 2 or day 5, 24 hours after miRNA transfection. cDNA was synthesized using the Superscript III Kit for mRNA (Invitrogen). For miRNA analysis, total RNA from d5 cells was subject to polyA addition and cDNA synthesis (Ncode kit, Invitrogen). Real-time PCR analyses for miRNAs were performed with FastStart Universal SYBR green (Roche) using universal reverse qPCR primer (Invitrogen) and forward primers with sequence identical to that of the mature miRNA sequence. Solaris mRNA assays (Dharmacon) were used for *Pten, CDKN1a, Bim,* and *Eomes* and 5'-FAM labeled probes were used for *Tbx21* and *Gapdh*, All qPCR was done using a realplex2 (Eppendorf).

Tbx21 sense 5′-CAACAACCCCTTTGCCAAAG-3′;

Tbx21 anti-sense 5′-TCCCCCAAGCAGTTGACAGT-3′;

Tbx21 probe FAM-5′- CCGGGAGAACTTTGAGTCCATGTACGC-3′-Tamra.

Gapdh sense 5'-CTCGTCCCGTAGACAAAATGG-3';

Gapdh antisense 5'-AATCTCCACTTTGCCACTGCA-3';

Gapdh probe FAM-5'-CGGATTTGGCCGTATTGGGCG-3'-Tamra.

Solexa Sequencing and Bioinformatics Analysis

Purified $CD4^+$ T cells were activated for 44 h under Th1 (10 ng/mL recombinant IL-12 (Peprotech)) or Th2 conditions. Total RNA was isolated using miRNeasy kit (Qiagen). Small (18-30 bp) RNA libraries were constructed as described previously (107) and samples were sequenced on an Illumina 1G Genome Analyzer.

Adaptor sequences were trimmed from sequence reads as described previously (82). All adaptor-extracted reads 15-30 nt in length were mapped to the mouse genome (UCSC mm8 assembly) and only sequences with perfect matches to the genome were used for further analysis. Mouse scRNA, snRNA, srpRNA and rRNA sequences annotations were compiled from the UCSC genome browser RepeatMasker track (108), and miRNA sequences were annotated using the miRanalzyer online database (http://web.bioinformatics.cicbiogune.es/microRNA/miRanalyser.php) (109). Mouse tRNA annotations were compiled from the Lowe lab tRNA database website (http://lowelab.ucsc.edu/GtRNAdb/credits-citation.html) (110) and mouse snoRNA annotations were compiled as described previously (111).

Luciferase Reporter assays

The full-length 3'UTR of *Tbx21* and *Eomes* were cloned into the psiCHECK-2 luciferase reporter construct (Promega). $CD4^+$ T cells were co-transfected on d4 of culture with reporter constructs and miRNA mimics or inhibitors. Luciferase

activity was measured 24h after transfection using the Dual Luciferase Reporter Assay System (Promega) and a FLUOstar Optima plate-reader (BMG Labtech).

ACKNOWLEDGEMENTS

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FIGURES

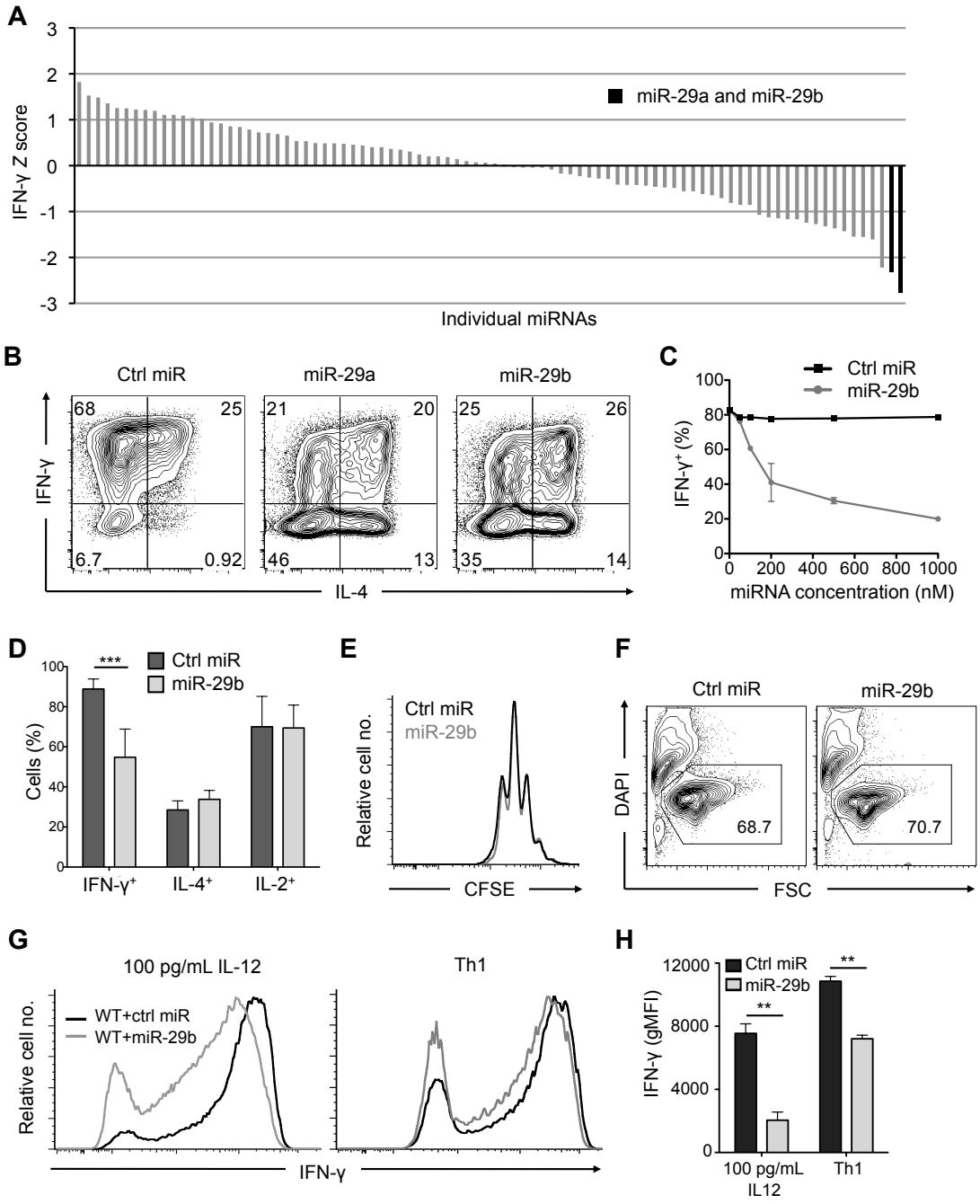

Figure 1.

Decreased proliferation and increased IFN-γ production by DGCR8-deficient CD4$^+$ T cells

(A) qPCR analysis of miRNA expression in FACS-sorted YFP^+ $CD4^+$ T cells cultured *in vitro* for 5 days. Bars represent miRNA expression relative to sno202; error bars represent range for replicate qPCR reactions.

(B) Proliferation of *Dgcr8$^{fl/fl}$ CD4-Cre R26-tdRFP* (gray lines) or *Dgcr8$^{+/fl}$ CD4-Cre R26-tdRFP* (black lines) RFP^+ $CD4^+$ T cells labeled with CFSE and analyzed by flow cytometry at the indicated times in culture.

(C-E) Cytokine production by restimulated YFP^+ $CD4^+$ T cells cultured in ThN or Th2 conditions (C), $CD4^+$ T cells cultured in Th1 conditions (D), and DGCR8-deficient YFP^+ $CD4^+$ T cells cultured in ThN conditions ± 5 μg/mL anti-IFN-γ (E). Data are representative of at least 3 independent experiments.

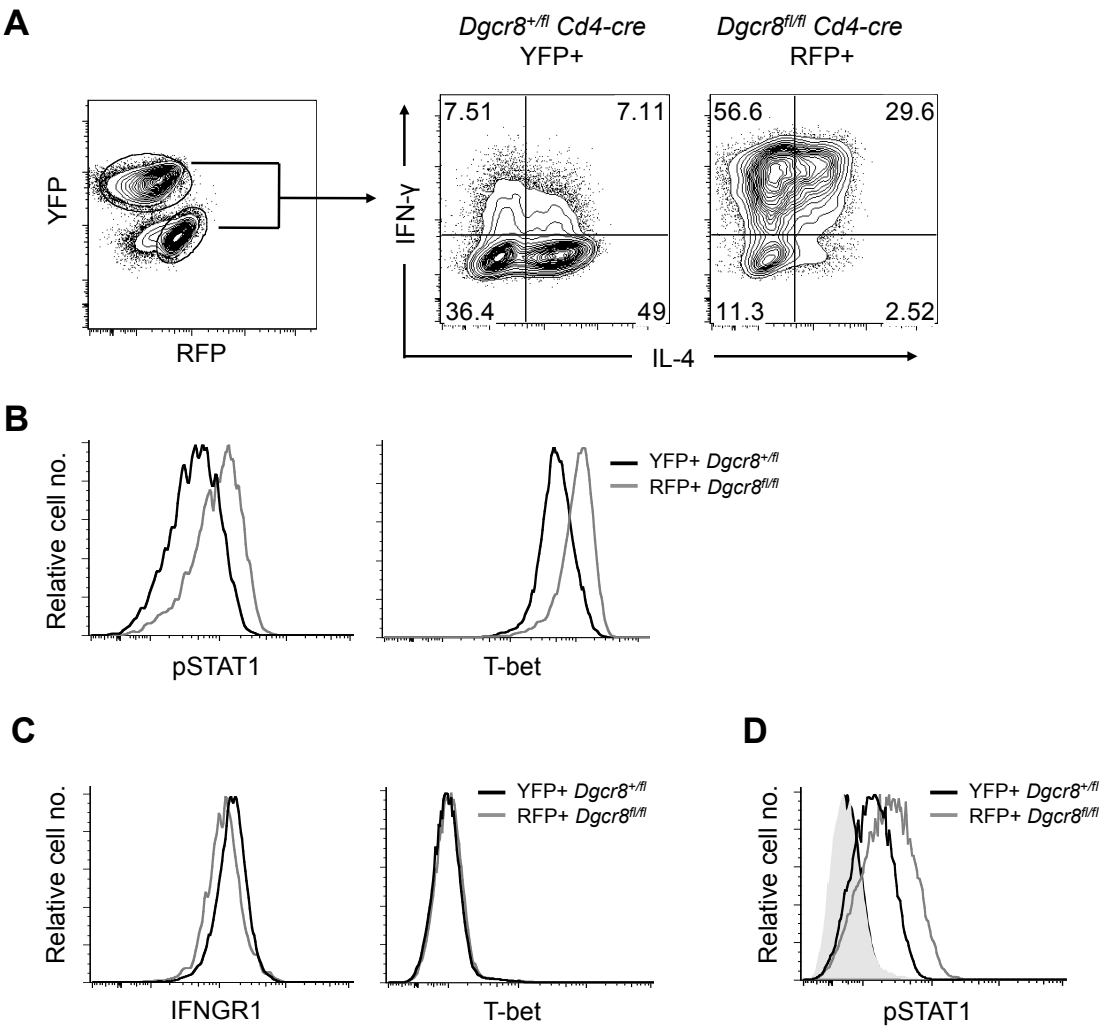

Figure 2.

DGCR8-deficient T cells exhibit cell-intrinsic defects in cytokine production and IFN-γ signaling

(A) Cytokine production of YFP^+ or RFP^+ $CD4^+$ T cells isolated from *Dgcr8*$^{fl/fl}$ *CD4-Cre R26-tdRFP* and *Dgcr8*$^{+/fl}$ *CD4-Cre R26-YFP* mice. YFP and RFP fluorescence (left panel) was used to distinguish *Dgcr8*$^{+/fl}$ and *Dgcr8*$^{fl/fl}$ cells, respectively (right panels). Cells were mixed in a 4:1 ratio (KO:control) and co-cultured in 10 u/mL IL-4. Data are representative of 3 independent experiments.

(B) Intracellular protein expression of co-cultured *Dgcr8$^{fl/fl}$ CD4-Cre R26-tdRFP* (Dgcr8 KO) RFP^{+} and *Dgcr8$^{+/fl}$ CD4-Cre R26-YFP* (Control) YFP^{+} cells after 68 hours of stimulation (anti-CD3/28)

(C) IFNGR1 and T-bet expression in freshly isolated CD4^{+} T cells.

(D) Phosphorylated STAT1 expression in freshly isolated CD4^{+} T cells ± 10 ng/mL IFN-γ for fifteen minutes. Shaded histograms represent *Dgcr8$^{fl/fl}$ CD4-Cre* RFP^{+} (gray) or *Dgcr8$^{+/fl}$ CD4-Cre* YFP^{+} (black) cells that were not treated with IFN-γ.

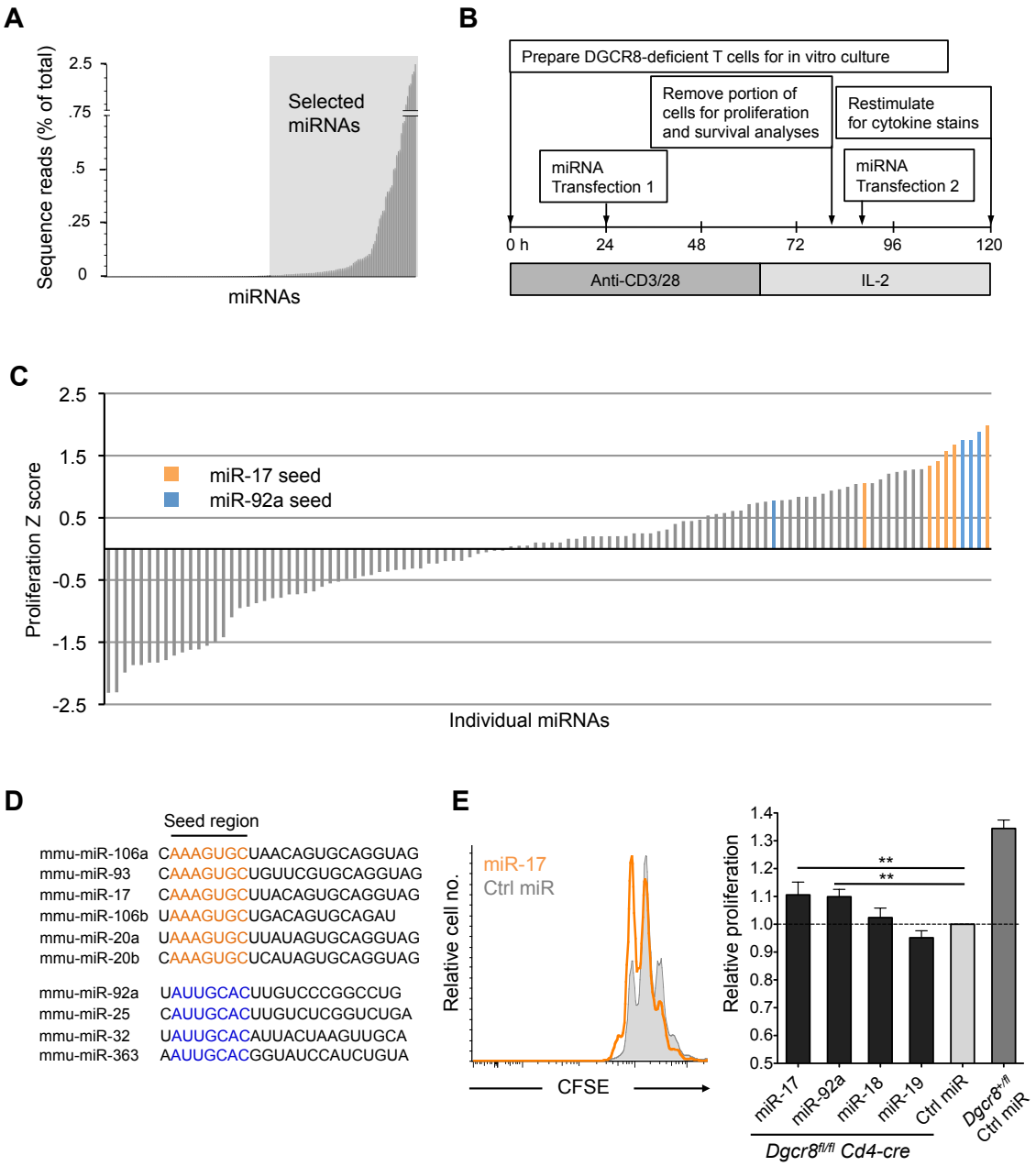

Figure 3.

A screen for miRNA function in DGCR8-deficient CD4$^+$ T cells reveals proliferation rescue by miR-17 and miR-92 family miRNAs

(A) miRNA expression from deep sequencing analysis of small RNAs in wildtype CD4$^+$ T cells stimulated *in vitro* for 44 hours. Shaded region marks miRNAs selected for screening. Unshaded region represents all miRNAs sequenced at

least once. Bars are average frequency of each miRNA among all genome-matching small RNA sequences from two independent small RNA libraries.

(B) Schematic of workflow for screening individual miRNAs for functional effects in DGCR8-deficient $CD4^+$ T cells (from *Dgcr8*$^{fl/fl}$ *CD4-Cre R26-tdRFP* mice).

(C) Proliferation index for each miRNA was determined (see methods) and used to calculate proliferation *Z* scores (*Z* = *x*-mean/SD where *x* represents the proliferation index for each individual miRNA). All miRNAs in the miR-17 and miR-92 seed families that were screened are highlighted.

(D) miR-17 and miR-92 seed family miRNAs (ordered from highest to lowest Z score).

(E) Proliferation of CFSE-labeled, RFP^+ cells 65 h post transfection with individual miR-17~92 cluster miRNAs or control miRNA (representative histograms in left panel). Proliferation index was determined as in (A) (see methods) and normalized to the proliferation index for cells transfected with control miRNA. Values are means ± SD from 5 independent transfections in 3 independent experiments; $^{**}p<0.01$; ANOVA Tukey's post-hoc test.

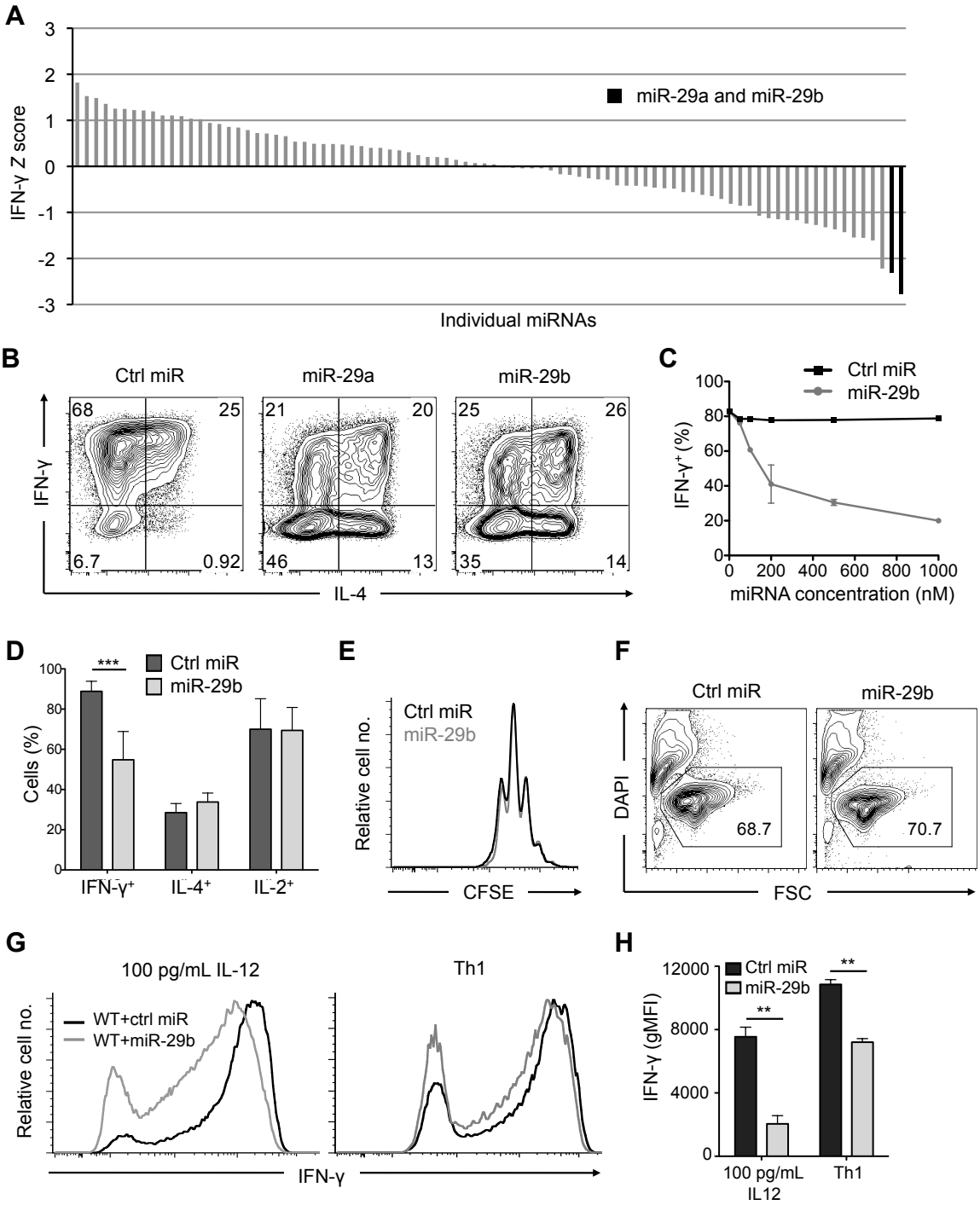

Figure 4.

MiR-29a and miR-29b rescue aberrant IFN-γ production by miRNA deficient CD4$^+$ T cells and repress IFN-γ production by wildtype Th1 cells.

(A) *Z* scores for frequency of IFN-γ production among restimulated DGCR8-deficient cells transfected with individual miRNAs as described in Figure 3B. MiRNAs with a proliferation score of $Z < -1.5$ or $Z > 1.5$ were not included in the IFN-γ analysis due to possible indirect effects of survival or proliferation on cytokine production.

(B) Intracellular cytokine stains for IFN-γ and IL-4 in transfected and restimulated DGCR8-deficient $CD4^+$ T cells. Data are representative of three independent experiments.

(C) Frequency of IFN-γ production among *Dgcr8*$^{fl/fl}$ *CD4-Cre* $CD4^+$ T cells transfected with increasing concentration of miR-29b. Data are representative of 2 independent experiments; error bars represent range from two independent transfections.

(D) Summary of cytokine production by miR-29b-transfected and restimulated *Dgcr8*$^{fl/fl}$ *CD4-Cre* $CD4^+$ T cells. Values are means ± SD from four biological replicates in two independent experiments; ***$p<0.001$; Student's two-tailed *t*-test.

(E-F) Proliferation analysis by CFSE dilution and viability analysis by DAPI exclusion of transfected *Dgcr8*$^{fl/fl}$ *CD4-Cre* cells on d5. Numbers indicate percentage of total events that are viable (F).

(G-H) IFN-γ production by restimulated, wildtype C57BL/6 $CD4^+$ cells cultured in 100 pg/mL IL-12 or Th1 conditions and transfected with miR-29b or control miRNA. Values in (H) represent mean ± SD from three independent transfections and data are representative of two independent experiments.

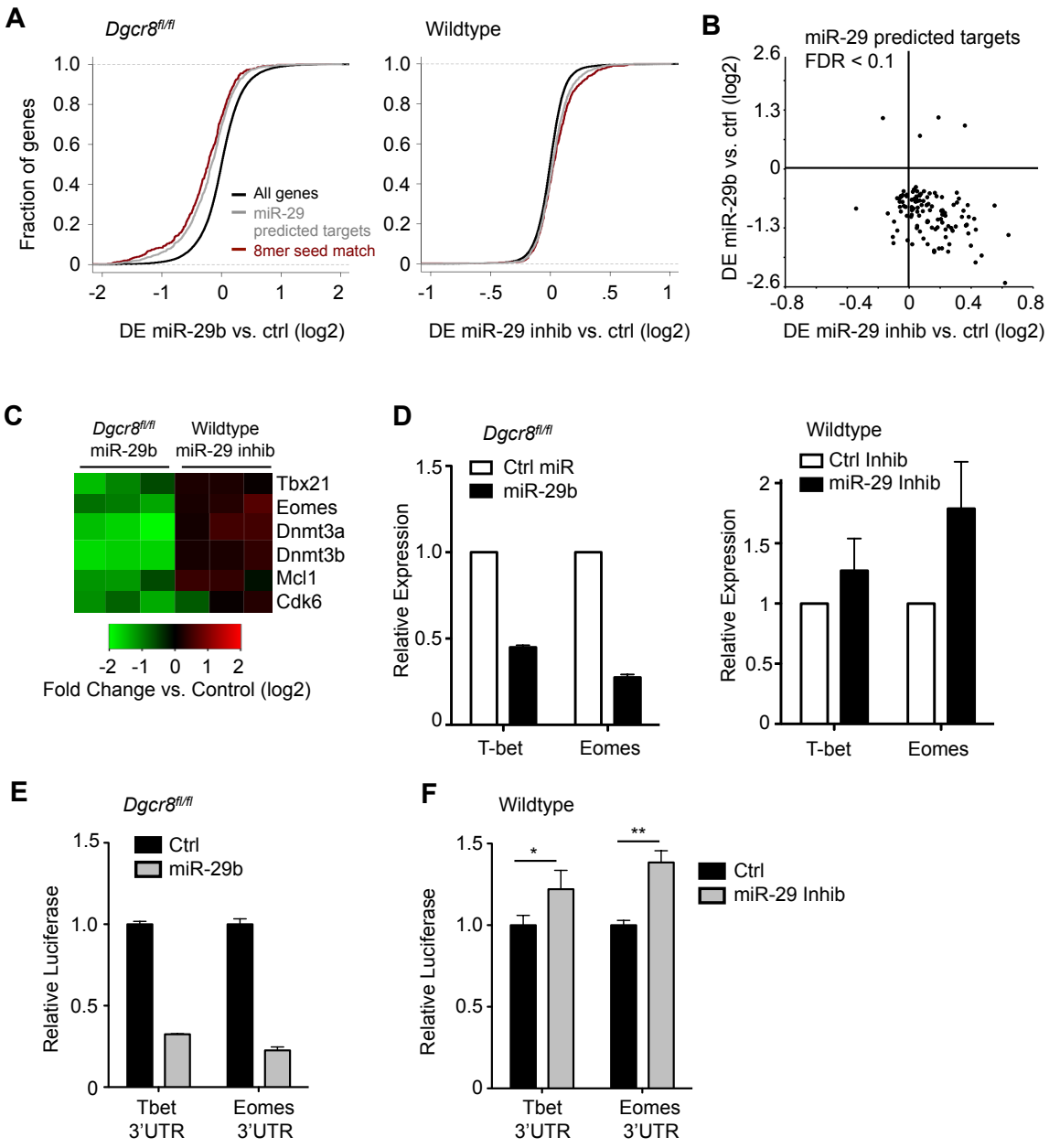

Figure 5.

Genome-wide analysis identifies T-bet and Eomes as miR-29 targets

(A) Cumulative distribution of mRNA expression changes from microarray data following miR-29b transfection in *Dgcr8*$^{fl/fl}$ *CD4-Cre* (Dgcr8 KO) CD4^{+} T cells (left panel) or miR-29 inhibitor transfection in C57BL/6 (wildtype) CD4^{+} T cells (right

panel). Negative values indicate downregulation of mRNA following transfection. Black lines represent the entire microarray gene set, gray lines represent the subset of genes that are Targetscan computationally predicted miR-29 targets, and red lines represent the subset of predicted targets that have 8mer binding sites with perfect complementary to the entire miR-29 seed sequence.

(B) Scatter plot of the change in mRNA expression following miR-29 transfection in *Dgcr8*$^{fl/fl}$ *CD4-Cre* cells (Y axis) vs. change in expression following miR-29 inhibitor transfection in wildtype cells (X axis). Each point represents a different gene array probe. Included are predicted miR-29 targets differentially expressed (FDR < 0.1) following miR-29 transfection.

(C) Heatmap representation of changes in gene expression following transfection for three independent biological samples. Scale is log2 fold change in array hybridization intensity compared to control transfected cells.

(D) Real-time qPCR validation of array data for miR-29 candidate targets of interest. mRNA expression was normalized to *Gapdh* and is presented relative to expression in control-transfected cells. Values are means ± SD from three independent biological samples.

(E-F) Primary *Dgcr8*$^{fl/fl}$ *CD4-Cre* (E) or wildtype (F) CD4^{+} T cells were co-transfected with a dual luciferase reporter and miR-29b or control miRNA (E); or miR-29 inhibitors or control inhibitor (F). Luciferase reporters contained the full length mouse 3'UTR of *Tbx21* or *Eomes.* Renilla luciferase activity was measured 24 hours after transfection and normalized to firefly luciferase activity. Values are relative to normalized luciferase activity in control transfected cells.

Data are representative of three independent experiments and values are means ± SD from three independent transfections.

*p<0.05, **p<0.01; ANOVA Tukey's post hoc test.

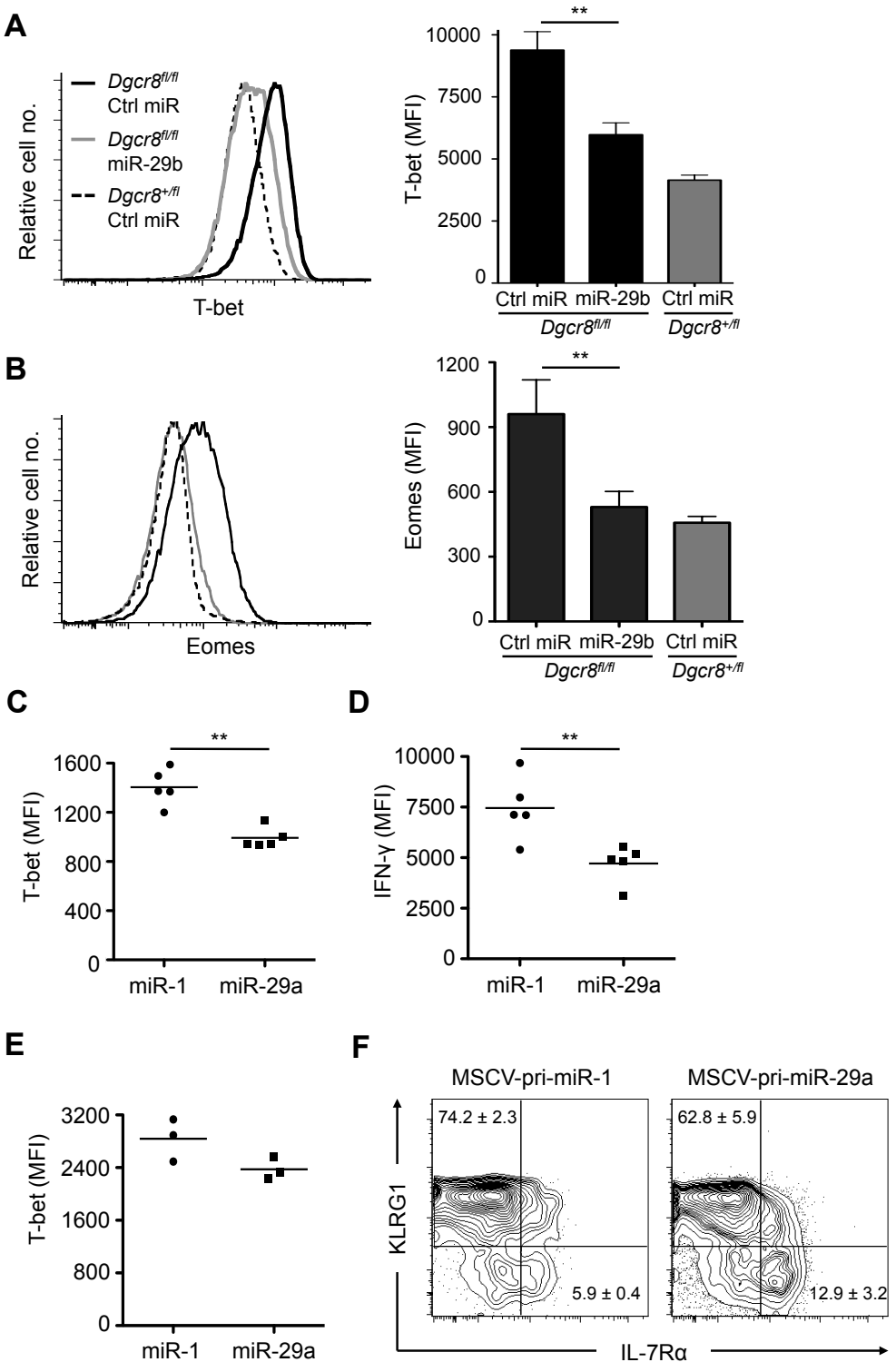

Figure 6.

miR-29 regulates T-box transcription factor protein expression in vitro and in vivo

(A-B) $CD4^+$ cells from *Dgcr8*$^{fl/fl}$ *CD4-Cre* mice (KO) or *Dgcr8*$^{+/fl}$ *CD4-Cre* mice (Control) were cultured in ThN conditions and stained intracellularly for T-bet and Eomes protein 24 hours after transfection with miR-29b or control miRNA. T-bet data is on day 2 and Eomes data is on day 5. Values are means ± SD from three independent transfections; ***p*<0.01; ANOVA Tukey's post hoc test.

(C) MFI of intracellular T-bet stains for $Thy1.1^+$, $CD45.1^+$ cells following adoptive transfer of $CD4^+$ SMARTA cells and LCMV infection. Transferred and transduced cells were identified by congenic CD45.1 and retroviral Thy1.1 expression respectively.

(D) MFI of intracellular IFN-γ for transduced and transferred $CD4^+$ SMARTA cells (as in (C)). Cells were stimulated with 1 μM gp61-80 peptide. Data represents IFN-γ MFI for TNF-α producing cells.

(E) MFI of intracellular T-bet in $CD8^+$ P14 cells following LCMV infection and analyzed as in (C).

(F) Transduced $CD8^+$ P14 cells from the same experiments as in (E) were analyzed for surface expression of KLRG1 and IL-7Rα. Values are mean frequency (%) ± SD; n=3; *p<0.05;* Student's two tailed *t*-test. Data in (C-F) are representative of two independent experiments; ***p*<0.01; Student's two tailed *t*-test.

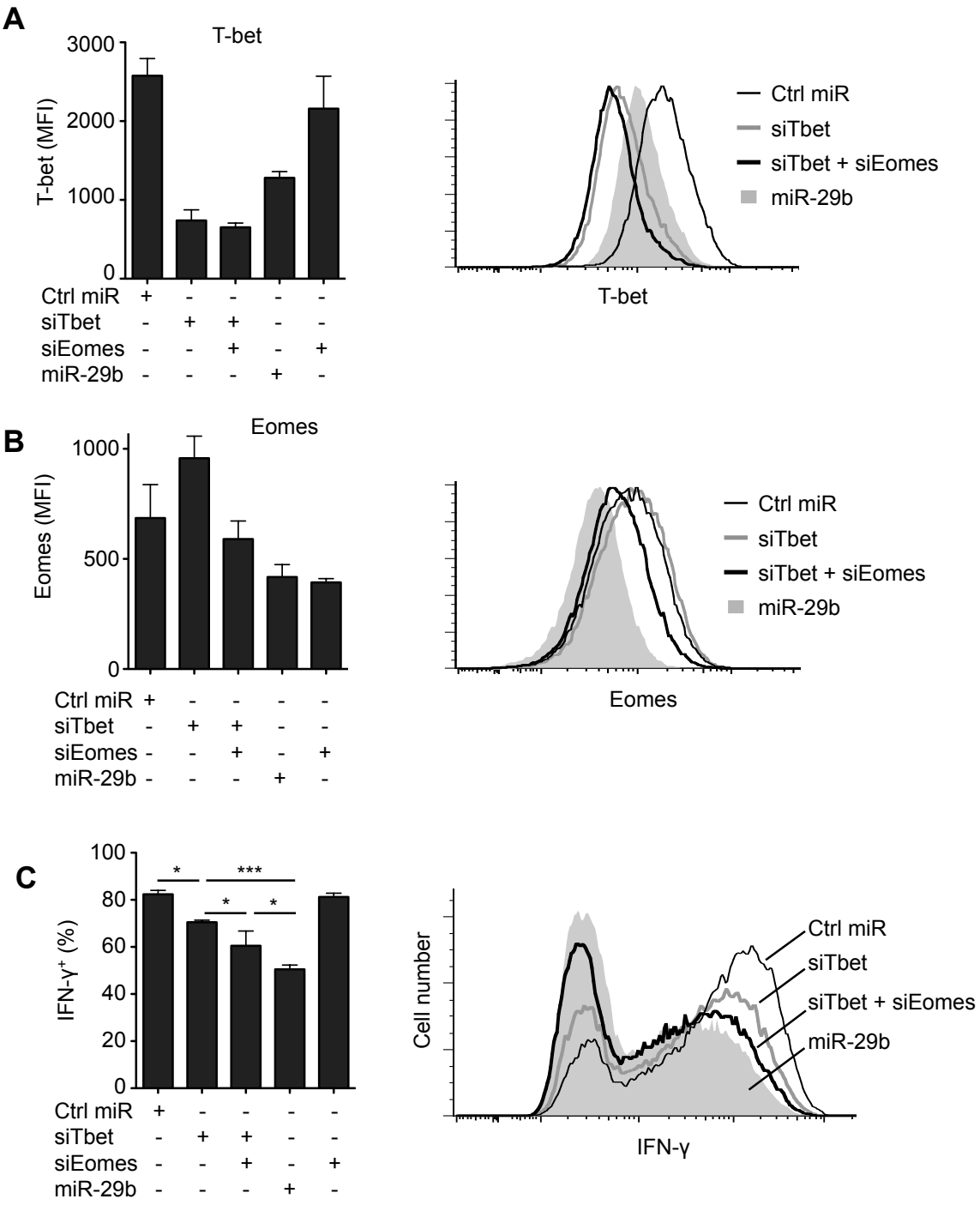

Figure 7.

miR-29-mediated repression of IFN-γ involves regulation of both T-bet and Eomes

(A-C) *Dgcr8*$^{fl/fl}$ *cd4-Cre* CD4^{+} T cells were transfected with control miRNA, miR-29b, siRNA targeting T-bet (siTbet), and/or siRNA targeting Eomes (siEomes). Cells were cultured in ThN conditions and transfected at 24 hours and 96 hours. T-bet (A), Eomes (B), and IFN-γ (C) protein expression was analyzed following restimulation at 120 hours. Values are means ± SD from 3 independent transfections, ***p<0.001, *p<0.05; ANOVA Tukey's post hoc test.

SUPPLEMENTAL FIGURES AND TABLES

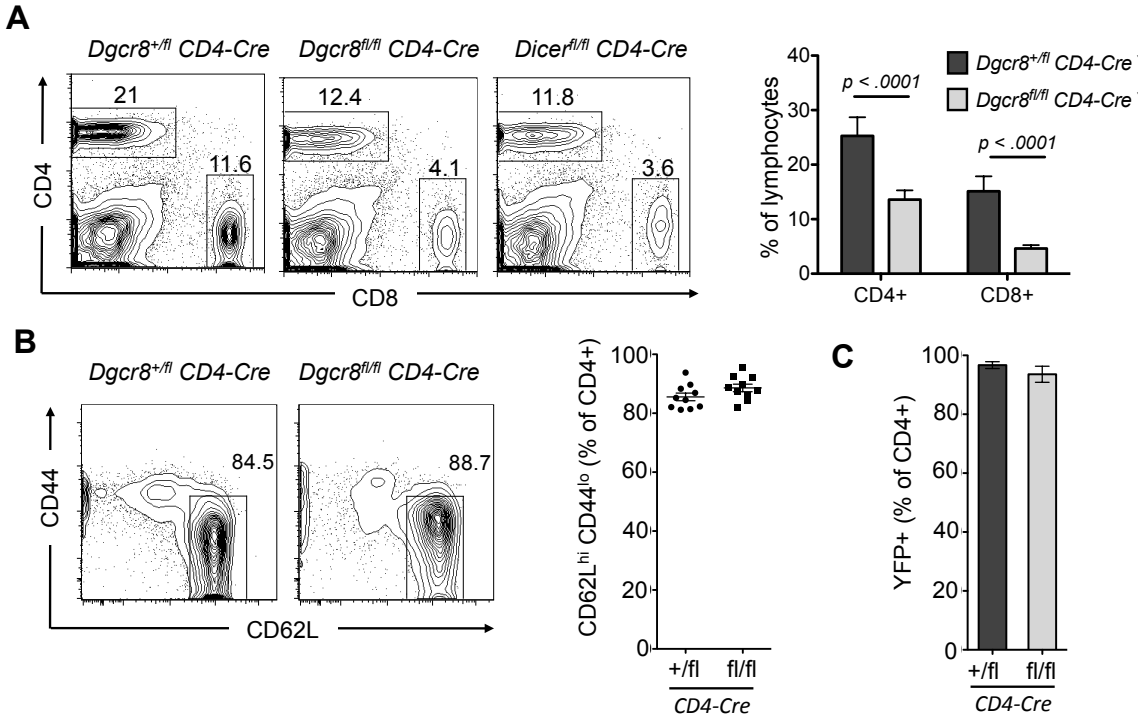

Figure S1 (related to Figure 1). Phenotype of cells isolated from the lymph nodes and spleens of *Dgcr8$^{fl/fl}$ CD4-Cre* mice.

(A) Total lymphocytes were isolated from lymph nodes and spleens of *Dgcr8$^{fl/fl}$ CD4-Cre, Dgcr8$^{+/fl}$ CD4-Cre,* or *Dicer$^{fl/fl}$ CD4-Cre* mice and immediately analyzed for surface expression of CD4 and CD8. Plots depict relative frequencies among all live lymphocytes. Bar graphs are means ± SD (n=10).

(B) The CD4^{+} cells among the freshly isolated lymphocytes obtained as in (A) from *Dgcr8$^{fl/fl}$ CD4-Cre, Dgcr8$^{+/fl}$ CD4-Cre* mice were further analyzed for cell surface expression of CD62L and CD44.

(C) $CD4^+$ cells from *Dgcr8*$^{fl/fl}$ *CD4-Cre R26-YFP* or *Dgcr8*$^{+/fl}$ *CD4-Cre R26-YFP* mice were analyzed for YFP expression (with YFP used as a marker for Cre expression and in this case, *Dgcr8* inactivation).

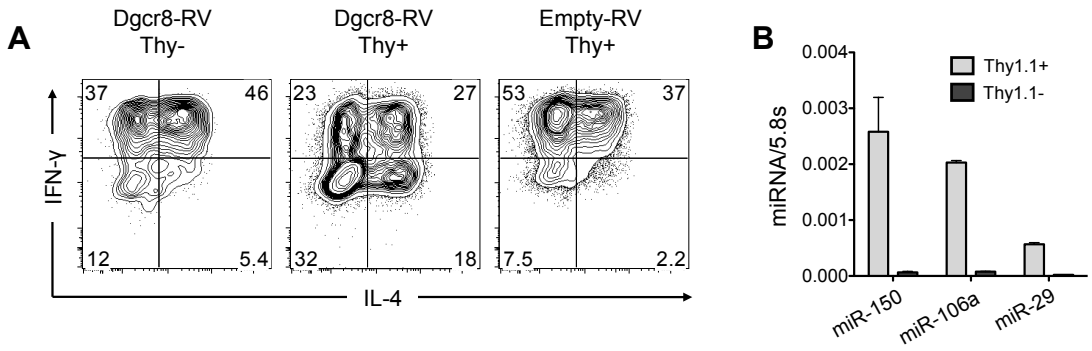

Figure S2 (related to Figure 2). Restoring miRNA expression rescues cytokine production defects of DGCR8-deficeint cells in a cell intrinsic manner

(A) $CD4^+$ T cells isolated from *Dgcr8$^{fl/fl}$ CD4-Cre R26-YFP* mice were transduced with MSCV-Dgcr8-Thy1.1 (Dgcr8-RV) or MSCV-Thy1.1 (Empty-RV) after 48 hours of stimulation. Cytokine analysis was performed on day 5 using Thy1.1 (Thy) expression to distinguish transduced and untransduced cells. Data are representative of 6 independent experiments.

(B) miRNA expression following re-introduction of DGCR8. $CD4^+$ T cells isolated from *Dgcr8$^{fl/fl}$ CD4-Cre R26-YFP* mice were transduced with MSCV-Dgcr8-Thy1.1 on day 2. Transduced ($Thy1.1^+$) and untransduced ($Thy1.1^-$) cells were isolated by FACS on day 5 and miRNA expression was analyzed by qPCR. Error bars represent range for replicate qPCR reactions.

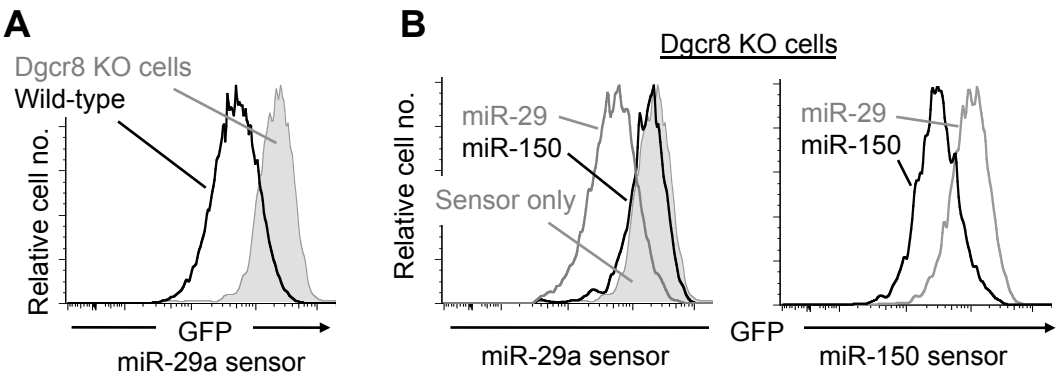

Figure S3 (related to Figure 3). Validating miRNA gain of function by transfection in primary helper T cells

(A) $CD4^+$ T cells were isolated from *Dgcr8$^{fl/fl}$ CD4-Cre* or wildtype mice and transduced with miR-29a sensor retrovirus on day 2 (see supplemental procedures for sensor description). Sensor GFP expression was analyzed on day 5. To control for possible differences in transduction efficiency between cultures, analysis was limited to cells with equal expression of human-CD25 that is independently transcribed from the sensor retrovirus and is not affected by miRNA activity.

(B) *Dgcr8$^{fl/fl}$ CD4-Cre R26-tdRFP* $CD4^+$ T cells were transduced with miR-29a sensor (left panel) or miR-150 sensor (right panel) on day 2. Cells were transfected with miRNA oligonucleotides on day 4 and analyzed 24 hours later as in (A).

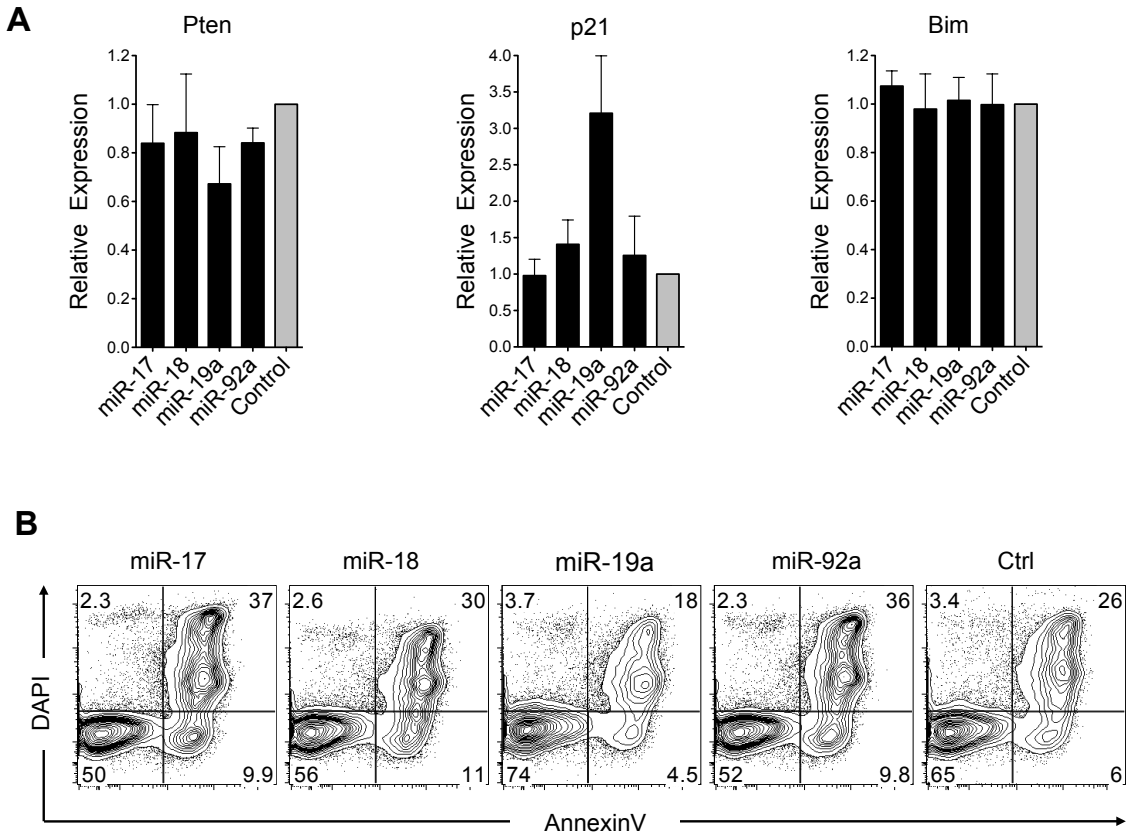

Figure S4 (related to Figure 3). Effects of miR-17~92 in DGCR8-defiecient helper T cells.

(A) Expression of previously validated miR-17~92 target mRNAs. DGCR8-deficient $CD4^+$ T cells were transfected with individual miRNAs of the miR-17~92 cluster. RNA was isolated 24 hours following transfection and subjected to qRT-PCR for the indicated genes. mRNA expression was normalized to Gapdh and is presented relative to expression in cells transfected with control miRNA. Values are means ± SD from 3 biological replicates.

(B) Viability analysis on day five of culture for DGCR8-deficient cells transfected with the miR-17~92 cluster miRNAs. Representative of 3 independent experiments.

	miRNA* sequence	No. miRNA* reads	Mature miRNA sequence	No. miRNA Reads
miR-29a	ACTGATTTCTTTTGGTGTTCAG	213	TAGCACCATCTGAAATCGGTTA	20157
miR-29b-1	GCTGGTTTCATATGGTGGTTTAGATTTA	53	TAGCACCATTTGAAATCAGTGTT	23808
miR-29b-2	CTGGTTTCACATGGTGGCTTAGATT	6	TAGCACCATTTGAAATCAGTGTT	23875
miR-29c	TGACCGATTTCTCCTGGTGTTC	8	TAGCACCATTTGAAATCGGTTA	89

Chromosome 6: mmu-miR-29a/29b-1

Chromosome 1: mmu-miR-29b-2/29c

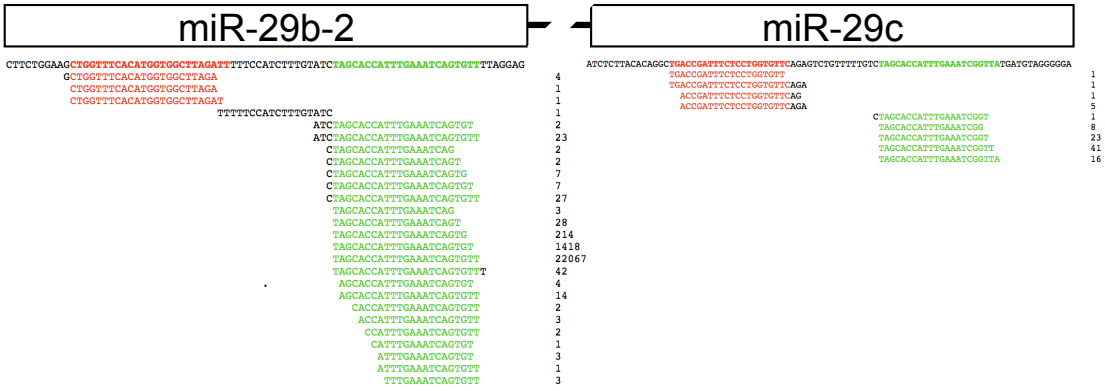

Figure S5 (related to Figure 4). Deep sequencing analysis of miR-29 cluster expression in activated helper T cells.

Small RNA reads mapping to the miR-29 sequence clusters on chromosome 1 and 6. Deep sequencing libraries of small (18-30 bp) RNAs were generated from $CD4^+$ T cells stimulated *in vitro* for 44 hours with anti-CD3 and anti-CD28. Read numbers were obtained from two independent small RNA libraries and represent only perfect matches to the miR-29 loci. Note that miR-29b sequence reads map to both the miR-29b-1 and the miR-29b-2 loci because they share identical mature miRNA sequences. However, the loci are distinguished by different miR-29b* sequences as well as the linked miR-29a or miR-29c sequences.

Table S1: Small RNA sequencing reads in activated helper T cells

microRNA	raw reads	% total sequencing reads	% total miRNA reads
mmu-let-7f	98968	2.567261803	10.76729269
mmu-miR-24	92476	2.136708376	8.434538373
mmu-miR-191	87600	2.245526475	9.363943875
mmu-miR-16	72662	1.785111856	7.286373422
mmu-miR-142-5p	61162	1.709625272	7.417711313
mmu-miR-142-3p	56784	1.309521095	5.163636743
mmu-let-7i	34753	0.994290971	4.356655896
mmu-miR-15b	28358	0.724668579	3.017309363
mmu-miR-19b	26201	0.590678718	2.298473913
mmu-miR-146a	25419	0.71594919	3.116495005
mmu-let-7d	24213	0.668810248	2.886892534
mmu-miR-423-3p	21876	0.566163491	2.371905691
mmu-let-7a	19743	0.506426819	2.112534567
mmu-miR-378	19679	0.500622766	2.079835702
mmu-miR-7a	18470	0.584127304	2.660968325
mmu-miR-150	17146	0.396516105	1.566011798
mmu-miR-15a	15932	0.415478973	1.746994725
mmu-miR-22	15885	0.426099249	1.815311003
mmu-miR-181a	15351	0.39811081	1.669534144
mmu-miR-425	13795	0.373303411	1.596760677
mmu-miR-29b	11879	0.307365421	1.287572887
mmu-miR-744	11516	0.309101588	1.317273637
mmu-let-7c	11347	0.287482645	1.19192964
mmu-let-7g	10369	0.270889923	1.13998213
mmu-miR-29a	8951	0.2349703	0.991094899
mmu-miR-155	7788	0.201806728	0.845957663
mmu-miR-19a	7446	0.166497606	0.644748977
mmu-miR-106b	6106	0.152526871	0.627919897
mmu-miR-423-5p	4871	0.131343508	0.560892175
mmu-let-7b	4245	0.106874791	0.441750205
mmu-miR-26a	4095	0.106332426	0.446181047
mmu-miR-467e	3890	0.098722724	0.409684121
mmu-miR-21	3782	0.089841516	0.360188061
mmu-miR-18a	3519	0.094841714	0.404951332
mmu-miR-93	3407	0.083378552	0.339644621
mmu-miR-17	3342	0.084130415	0.347724978
mmu-miR-363	3189	0.081476007	0.339234563
mmu-miR-103	3095	0.082315506	0.349344807
mmu-miR-27b	2885	0.069475884	0.280634494
mmu-miR-148a	2685	0.073868562	0.318291174

mmu-miR-182	2512	0.064280351	0.267850289
mmu-miR-183	2095	0.053011362	0.219678916
mmu-miR-484	1946	0.046422396	0.1865687
mmu-miR-185	1882	0.052545699	0.227896912
mmu-miR-130b	1747	0.045262951	0.189752216
mmu-miR-25	1632	0.038868428	0.156049339
mmu-miR-30b	1588	0.039864828	0.164525743
mmu-miR-148b	1571	0.041678332	0.176689842
mmu-miR-652	1541	0.038019242	0.155552095
mmu-miR-106a	1530	0.038011252	0.156050057
mmu-miR-27a	1496	0.03518446	0.140284732
mmu-miR-374	1459	0.034607626	0.138661354
mmu-miR-467c	1280	0.030257984	0.121008978
mmu-miR-23a	1241	0.028970909	0.115031189
mmu-miR-20a	1218	0.029347532	0.118567234
mmu-miR-30d	1166	0.029284036	0.120910954
mmu-miR-96	1092	0.025379264	0.100543354
mmu-miR-720	1074	0.026403959	0.107813433
mmu-miR-296-3p	1040	0.026762861	0.111839211
mmu-miR-30e	859	0.021752112	0.090189421
mmu-miR-872	858	0.024814187	0.10921355
mmu-miR-30c	851	0.022061761	0.092501672
mmu-miR-181c	847	0.021557792	0.089583478
mmu-miR-98	815	0.022270674	0.09567509
mmu-miR-186	815	0.020458609	0.084458556
mmu-miR-467d	756	0.017741739	0.070678873
mmu-miR-28	749	0.018103347	0.073287419
mmu-miR-342-3p	745	0.017598357	0.070336816
mmu-miR-132	713	0.016757165	0.066786477
mmu-miR-20b	662	0.015637918	0.062527846
mmu-miR-322	642	0.015718948	0.064047428
mmu-miR-467a	640	0.01502943	0.05989981
mmu-miR-31	604	0.017609637	0.077759403
mmu-miR-340-5p	597	0.015593042	0.065612843
mmu-miR-500	590	0.01683278	0.073695656
mmu-miR-194	561	0.014041849	0.057866021
mmu-miR-32	560	0.014121439	0.058438039
mmu-miR-181b	513	0.012816402	0.052792056
mmu-miR-320	512	0.014124258	0.060932468
mmu-miR-92a	511	0.01218473	0.048977255
mmu-miR-126-3p	493	0.011555278	0.045984499
mmu-miR-210	479	0.011984161	0.049375465
mmu-miR-151-3p	473	0.011879702	0.049066322
mmu-miR-193	456	0.011089269	0.045022641
mmu-miR-141	445	0.011671985	0.049237674

mmu-miR-26b	360	0.009278999	0.03882326
mmu-miR-101b	342	0.008526949	0.035095939
mmu-miR-140	332	0.008129419	0.033151005
mmu-miR-669c	327	0.008132723	0.033406157
mmu-miR-700	326	0.008265292	0.034306878
mmu-miR-23b	312	0.007122442	0.027946326
mmu-miR-674	305	0.007836226	0.032713816
mmu-miR-296-5p	282	0.006387991	0.024927873
mmu-miR-421	267	0.007442899	0.032255045
mmu-miR-298	259	0.007818322	0.035001453
mmu-miR-361	240	0.006109813	0.025418183
mmu-miR-181d	223	0.005331153	0.021447547
mmu-miR-33	196	0.004698577	0.018956867
mmu-miR-342-5p	191	0.005102172	0.021695552
mmu-miR-128	184	0.00487025	0.020647731
mmu-miR-297a	183	0.004918364	0.020972319
mmu-miR-212	180	0.004494766	0.018500624
mmu-miR-339-3p	162	0.004008636	0.016449225
mmu-miR-532-5p	156	0.003784403	0.015344815
mmu-miR-18b	155	0.003681506	0.014784634
mmu-miR-328	153	0.003690456	0.014918382
mmu-miR-101a	144	0.00357933	0.014698236
mmu-miR-151-5p	120	0.003033793	0.012565059
mmu-miR-99b	104	0.002502372	0.010128369
mmu-miR-192	95	0.002307812	0.009364499
mmu-miR-669a	95	0.002355926	0.009689088
mmu-miR-669f	95	0.002126347	0.008264709
mmu-miR-805	90	0.002277837	0.009465333
mmu-miR-326	88	0.002110188	0.008503404
mmu-miR-200c	84	0.002100697	0.008653103
mmu-miR-301a	79	0.001969629	0.008100645
mmu-miR-149	75	0.001865951	0.007665984
mmu-miR-107	68	0.00198032	0.008740583
mmu-miR-1198	68	0.001674215	0.006841411
mmu-miR-339-5p	67	0.001440791	0.005445326
mmu-miR-466g	67	0.001876403	0.008147994
mmu-miR-350	65	0.001563629	0.006337819
mmu-miR-501-3p	63	0.001673673	0.007124858
mmu-miR-144	62	0.001868954	0.00836251
mmu-miR-147	62	0.002046574	0.009490594
mmu-miR-324-5p	61	0.001489385	0.006085769
mmu-miR-297b-5p	56	0.001417183	0.005898537
mmu-miR-532-3p	54	0.001296627	0.005228789
mmu-miR-450a-5p	47	0.001146097	0.004659874
mmu-miR-671-5p	46	0.001095159	0.004395988

mmu-miR-466k	46	0.001084407	0.004355351
mmu-miR-29c	44	0.00122875	0.005329117
mmu-miR-449a	42	0.001221061	0.005385705
mmu-miR-466d-5p	42	0.000968957	0.003847644
mmu-miR-466b-3-3p	41	0.000870926	0.003291578
mmu-miR-466i	39	0.000838669	0.003169667
mmu-miR-146b	34	0.000808695	0.003270501
mmu-miR-297c	33	0.000809716	0.003302909
mmu-miR-200a	33	0.000781304	0.003152705
mmu-miR-362-5p	32	0.00074214	0.002961864
mmu-miR-335-5p	31	0.000914895	0.004085834
mmu-miR-365	31	0.000732409	0.002953636
mmu-miR-379	30	0.000756977	0.003132134
mmu-miR-188-5p	30	0.000722678	0.002945407
mmu-miR-301b	29	0.000711926	0.00290477
mmu-let-7e	29	0.000712947	0.002937179
mmu-miR-466a-5p	26	0.000677867	0.002875971
mmu-miR-466d-3p	25	0.000537609	0.002031838
mmu-miR-466h	25	0.000649455	0.002725766
mmu-miR-669b	25	0.000637682	0.002652721
mmu-miR-125a-5p	25	0.000526856	0.001991201
mmu-miR-671-3p	25	0.000585723	0.002356427
mmu-miR-122	24	0.000570105	0.002311676
mmu-miR-503	24	0.000623085	0.002640379
mmu-miR-542-3p	24	0.000593652	0.002457766
mmu-miR-877	24	0.000670178	0.002932559
mmu-miR-345-3p	23	0.00056524	0.002307562
mmu-miR-188-3p	22	0.000620262	0.002701081
mmu-miR-362-3p	22	0.000561395	0.002335856
mmu-miR-874	22	0.000473096	0.001788018
mmu-miR-211	20	0.000518387	0.002173309
mmu-miR-690	20	0.000594913	0.002648102
mmu-miR-429	20	0.000478201	0.001950059
mmu-miR-541	20	0.000489974	0.002023104
mmu-miR-466c-5p	18	0.000476399	0.00204317
mmu-miR-200b	17	0.00042444	0.001746875
mmu-miR-195	16	0.00034407	0.001300376
mmu-miR-331-3p	16	0.000172035	0.000650188
mmu-miR-714	16	0.000426482	0.001811692
mmu-miR-138	15	0.000311813	0.001178466
mmu-miR-451	14	0.000290309	0.001097193
mmu-miR-30a	12	0.000258052	0.000975282
mmu-miR-345-5p	12	0.000258052	0.000975282
mmu-miR-130a	12	0.0002473	0.000934646
mmu-miR-152	12	0.000323826	0.001409439

mmu-miR-330	12	0.0002473	0.000934646
mmu-miR-501-5p	12	0.0002473	0.000934646
mmu-miR-331-5p	10	0.00027391	0.001177961
mmu-miR-139-5p	10	0.000204291	0.000772098
mmu-miR-221	9	0.000193539	0.000731462
mmu-miR-205	9	0.000182787	0.000690825
mmu-miR-1187	8	0.000172035	0.000650188
mmu-miR-219	8	0.000172035	0.000650188
mmu-miR-203	8	0.000161283	0.000609551
mmu-miR-542-5p	7	0.00015053	0.000568915
mmu-miR-574-5p	7	0.00015053	0.000568915
mmu-miR-99a	7	0.00015053	0.000568915
mmu-miR-450a-3p	7	0.000139778	0.000528278
mmu-miR-450b-3p	7	0.000139778	0.000528278
mmu-miR-466j	7	0.000139778	0.000528278
mmu-miR-466b-5p	6	0.000118274	0.000447004
mmu-miR-486	6	0.000118274	0.000447004
mmu-miR-497	6	0.000118274	0.000447004
mmu-miR-672	6	0.000118274	0.000447004
mmu-miR-100	5	0.000107522	0.000406368
mmu-miR-467b	5	0.000166388	0.000771593

Table S2: Summary of *Z* scores for rescue screens

Proiferation *Z* scores	
miRNA	***Z* score**
mmu-let-7a	1.28
mmu-let-7b	-0.49
mmu-let-7c	0.29
mmu-let-7d	1.21
mmu-let-7f	0.20
mmu-let-7g	-0.84
mmu-let-7i	-0.24
mmu-miR-101b	0.05
mmu-miR-103	-0.22
mmu-miR-106a	1.98
mmu-miR-106b	1.41
mmu-miR-125a-5p	NA
mmu-miR-126-3p	-0.36
mmu-miR-128	0.20
mmu-miR-130b	1.12
mmu-miR-132	-1.62
mmu-miR-140	0.20
mmu-miR-141	0.25
mmu-miR-142-3p	0.31
mmu-miR-142-5p	1.00
mmu-miR-144	0.89
mmu-miR-146a	-0.22
mmu-miR-147	-2.31
mmu-miR-148a	-0.55
mmu-miR-148b	-0.47
mmu-miR-151-3p	-0.08
mmu-miR-155	-1.87
mmu-miR-15a	-1.79
mmu-miR-15b	-1.56
mmu-miR-16	-0.93
mmu-miR-17	1.58
mmu-miR-181a	0.54
mmu-miR-181b	0.84
mmu-miR-181c	1.26
mmu-miR-182	0.10
mmu-miR-183	-0.42
mmu-miR-185	0.05
mmu-miR-186	0.45
mmu-miR-18a	0.46
mmu-miR-191	0.79
mmu-miR-193	-0.79
mmu-miR-194	-0.79

IFN-γ *Z* scores	
miRNA	***Z* score**
mmu-let-7a	-0.56
mmu-let-7b	0.66
mmu-let-7c	1.02
mmu-let-7d	1.82
mmu-let-7f	0.10
mmu-let-7g	0.49
mmu-let-7i	0.25
mmu-miR-101b	-1.61
mmu-miR-103	-0.62
mmu-miR-106b	0.54
mmu-miR-126-3p	-0.86
mmu-miR-128	-0.09
mmu-miR-130b	1.11
mmu-miR-140	-0.42
mmu-miR-141	-1.15
mmu-miR-142-3p	-0.48
mmu-miR-142-5p	0.94
mmu-miR-144	-0.29
mmu-miR-146a	-0.42
mmu-miR-148a	-1.37
mmu-miR-148b	-2.22
mmu-miR-151-3p	-0.23
mmu-miR-16	0.44
mmu-miR-181a	0.07
mmu-miR-181b	-0.65
mmu-miR-181c	-0.86
mmu-miR-182	0.20
mmu-miR-183	1.19
mmu-miR-185	-0.41
mmu-miR-186	-0.56
mmu-miR-18a	0.69
mmu-miR-191	-0.48
mmu-miR-193	-0.81
mmu-miR-194	-1.55
mmu-miR-19a	1.48
mmu-miR-19b	0.86
mmu-miR-200c	-1.24
mmu-miR-205	0.72
mmu-miR-20a	0.14
mmu-miR-20b	0.04
mmu-miR-21	1.09
mmu-miR-22	-1.27

mmu-miR-19a	0.10	mmu-miR-24	-0.01
mmu-miR-19b	-0.73	mmu-miR-26a	0.19
mmu-miR-200c	-0.71	mmu-miR-26b	-1.17
mmu-miR-205	0.25	mmu-miR-27a	0.48
mmu-miR-20a	1.33	mmu-miR-27b	0.20
mmu-miR-20b	1.05	mmu-miR-296-5p	-0.28
mmu-miR-21	1.24	mmu-miR-297a	1.21
mmu-miR-210	NA	mmu-miR-298	-1.32
mmu-miR-212	-1.72	mmu-miR-29a	-2.31
mmu-miR-22	-0.37	mmu-miR-29b	-2.76
mmu-miR-23a	-1.50	mmu-miR-301a	0.53
mmu-miR-24	0.78	mmu-miR-30b	0.31
mmu-miR-25	1.75	mmu-miR-30c	0.35
mmu-miR-26a	0.25	mmu-miR-30d	1.53
mmu-miR-26b	0.16	mmu-miR-30e	1.11
mmu-miR-27a	-0.53	mmu-miR-31	-0.44
mmu-miR-27b	0.42	mmu-miR-322	0.84
mmu-miR-28	-1.83	mmu-miR-326	-1.43
mmu-miR-296-3p	-1.99	mmu-miR-328	0.92
mmu-miR-296-5p	-0.87	mmu-miR-33	0.40
mmu-miR-297a	0.40	mmu-miR-340-5p	1.22
mmu-miR-298	-0.10	mmu-miR-342-3p	0.49
mmu-miR-29a	0.21	mmu-miR-342-5p	-0.71
mmu-miR-29b	0.45	mmu-miR-361	0.49
mmu-miR-301a	1.06	mmu-miR-363	-1.55
mmu-miR-30b	-0.34	mmu-miR-374	0.45
mmu-miR-30c	0.58	mmu-miR-375	0.37
mmu-miR-30d	1.04	mmu-miR-378	0.40
mmu-miR-30e	0.00	mmu-miR-421	0.06
mmu-miR-31	-0.05	mmu-miR-423-3p	-0.26
mmu-miR-32	1.75	mmu-miR-466g	0.71
mmu-miR-322	0.10	mmu-miR-466l	-0.17
mmu-miR-326	-0.95	mmu-miR-467a	1.25
mmu-miR-328	0.76	mmu-miR-467c	0.00
mmu-miR-33	0.16	mmu-miR-467e	1.25
mmu-miR-340-5p	0.62	mmu-miR-500	0.79
mmu-miR-342-3p	0.96	mmu-miR-532-5p	0.01
mmu-miR-342-5p	-0.44	mmu-miR-652	-1.13
mmu-miR-361	0.20	mmu-miR-674	-1.07
mmu-miR-363	0.77	mmu-miR-720	-0.02
mmu-miR-374	-0.20	mmu-miR-744	-0.19
mmu-miR-375	-0.19	mmu-miR-7a	-1.17
mmu-miR-378	0.04	mmu-miR-872	0.02
mmu-miR-421	0.10	mmu-miR-9	-0.45
mmu-miR-423-3p	-0.61	mmu-miR-96	1.04
mmu-miR-423-5p	-1.51	mmu-miR-98	1.36

mmu-miR-425	-1.87
mmu-miR-466g	0.61
mmu-miR-466l	0.94
mmu-miR-467a	0.10
mmu-miR-467c	0.84
mmu-miR-467e	0.00
mmu-miR-500	1.28
mmu-miR-532-5p	0.74
mmu-miR-652	-0.19
mmu-miR-669c	-1.83
mmu-miR-674	-0.24
mmu-miR-700	-2.31
mmu-miR-720	-0.33
mmu-miR-744	-0.73
mmu-miR-7a	-0.30
mmu-miR-872	0.56
mmu-miR-9	0.84
mmu-miR-92a	1.87
mmu-miR-93	1.68
mmu-miR-96	-0.32
mmu-miR-98	0.47

NA: Not Analyzed (too few cells for analysis)

Table S3: Differential gene expression following miR-29 transfection or inhibition (Targetscan predicted targets with FDR < 0.1**)**

Gene/Probe	log2 fold change KO	log2 fold change KO
Gtf3c4	-1.260467933	-1.260467933
Dnmt3b	-1.691815863	-1.691815863
Dnmt3a	-1.660551488	-1.660551488
Dynlt1	-1.104397068	-1.104397068
Kctd3	-1.161096319	-1.161096319
Klhdc3	-1.337999686	-1.337999686
Ifi30	-2.513086755	-2.513086755
Zbtb34	-1.421228115	-1.421228115
Bak1	-1.832409357	-1.832409357
Fbn1	-1.635705506	-1.635705506
Txndc4	-1.909335826	-1.909335826
Tdg	-1.7981937	-1.7981937
6330442E10Rik	-1.293733407	-1.293733407
Narf	-1.36323674	-1.36323674
Hiatl1	-1.15801911	-1.15801911
Chic2	-1.302848153	-1.302848153
Arsb	-1.378663246	-1.378663246
Zbtb5	-1.248986735	-1.248986735
Gng12	-0.980731246	-0.980731246
Erp29	-1.572865208	-1.572865208
Blmh	-1.328681228	-1.328681228
Erlin2	-0.709053267	-0.709053267
Entpd7	-1.254700389	-1.254700389
Fem1b	-1.098869383	-1.098869383
Slc31a1	-0.940776933	-0.940776933
Parg	-1.033387141	-1.033387141
Dcakd	-2.061489661	-2.061489661
Tbc1d7	-0.726407525	-0.726407525
Nanos1	-1.14739683	-1.14739683
Ing4	-1.05555469	-1.05555469
Kctd5	-0.938038435	-0.938038435
Hmgn3	-1.357890193	-1.357890193
Larp5	-0.798474689	-0.798474689
BC043098	-0.56641746	-0.56641746
Cbx2	-0.716053485	-0.716053485
Kifc2	1.136599241	1.136599241
Kdelc1	-0.677206788	-0.677206788
Pde7a	-0.704984145	-0.704984145
Dgkh	-0.91065056	-0.91065056
Csda	-1.498920033	-1.498920033
1110012D08Rik	0.731900642	0.731900642
Map4k4	-0.968141785	-0.968141785

Nfia	-0.796596702	-0.796596702
Eomes	-1.05635219	-1.05635219
Zfp91	-1.502908672	-1.502908672
Wdr40a	-0.852675689	-0.852675689
Arsb	-1.301874052	-1.301874052
Mfap3	-0.770688183	-0.770688183
Hmgcs1	-1.010414948	-1.010414948
Gbp6	-0.814035509	-0.814035509
Bdh1	-1.661061219	-1.661061219
Hip1	-0.794948268	-0.794948268
Nkiras2	-0.909459972	-0.909459972
Dnmt3a	-1.004422315	-1.004422315
Nfia	-0.790711778	-0.790711778
Setd8	-0.644282516	-0.644282516
Unc84a	-1.029181303	-1.029181303
Lamc1	-1.455941373	-1.455941373
Mybl2	-1.535139176	-1.535139176
Blmh	-1.268544104	-1.268544104
Tmem164	-1.172610533	-1.172610533
Icos	-0.653442534	-0.653442534
Luzp1	-1.054643211	-1.054643211
Mxd1	-1.182028613	-1.182028613
Lmtk2	-0.576254238	-0.576254238
Fem1b	-1.287511522	-1.287511522
Tnfaip1	-0.492409865	-0.492409865
Tdg	-1.823292312	-1.823292312
Arrdc4	0.957604785	0.957604785
Dot1l	-0.824444358	-0.824444358
Sms	-0.655505205	-0.655505205
Cav2	-1.229249157	-1.229249157
Ccnyl1	-0.649448769	-0.649448769
Gas7	-1.115214579	-1.115214579
Adam19	-1.334720207	-1.334720207
Chfr	-0.600511594	-0.600511594
Pdhx	-0.398487414	-0.398487414
Stx1a	1.122736948	1.122736948
Tcfeb	-0.489953887	-0.489953887
Usp52	-0.714091133	-0.714091133
Camk1d	-0.502848139	-0.502848139
Kbtbd8	-0.721425064	-0.721425064
Fbn1	-0.679719977	-0.679719977
Luzp1	-0.840392853	-0.840392853
Kdelc1	-0.581697797	-0.581697797
Mlf1	-1.659684736	-1.659684736
Dpysl2	-0.872543122	-0.872543122
Nanos1	-0.696117704	-0.696117704

Syncrip	-0.872552162	-0.872552162
Bdh1	-1.749605193	-1.749605193
Purg	-0.522414545	-0.522414545
Tmem183a	-0.658232644	-0.658232644
Ddef2	-0.537509282	-0.537509282
Ctnnbip1	-0.924088788	-0.924088788
Itgb1	-1.029680023	-1.029680023
Mlxip	-0.489487399	-0.489487399
Ddx3x	-0.600927094	-0.600927094
9530068E07Rik	-0.607233528	-0.607233528
Nasp	-0.836812552	-0.836812552
Sms	-0.548185195	-0.548185195
Rod1	-0.909272383	-0.909272383
Dolpp1	-0.623188	-0.623188
Lasp1	-0.595975235	-0.595975235
Dtx4	-0.451633655	-0.451633655
Nfia	-0.739539417	-0.739539417
Cbx6	-0.603267003	-0.603267003
Ankrd57	-0.581984086	-0.581984086
Pcyt1b	-0.806634138	-0.806634138
Gtlf3b	-0.920114213	-0.920114213
Hmgcs1	-0.836677399	-0.836677399
Ak3	-1.752957612	-1.752957612
4931406P16Rik	-0.526916966	-0.526916966
Hip1	-0.620364446	-0.620364446
Zfp91	-0.93094367	-0.93094367

CHAPTER 3:

MicroRNA-29 Promotes Expression of Tumor Necrosis Factor

ABSTRACT

Tumor necrosis factor-alpha (TNF) is a multi-functional cytokine that plays a role in many biological processes including inflammation, autoimmunity, and host defense. TNF is produced by many cell types and there is growing evidence that microRNAs (miRNAs) can contribute significantly to the regulation of TNF production in cells of the innate immune system. However, little is known about miRNA-mediated TNF regulation in other cell types. We carried out a functional screen for miRNA effects on TNF production in helper T cells and found that miR-29a and miR-29b individually promoted TNF expression in these cells. This increase in TNF expression involved both transcriptional and post-transcriptional effects mediated by the TNF promoter and 3'UTR, respectively. The effect of miR-29 on the 3'UTR of TNF required the AU-rich element (ARE) of this 3'UTR, indicating the ability of miR-29 to regulate ARE-mediated decay of the TNF transcript. Finally, miR-29 also promoted TNF production in RAW 264.7 cells in vitro and in primary $CD8^+$ cells in an in vivo infection model. Taken together, these findings support a role for miR-29 in regulating TNF production by cells of both the innate and adaptive immune system.

INTRODUCTION

Tumor necrosis factor (TNF) is a multi-functional cytokine that signals to many cell types and plays important roles in both healthy immunity and inflammatory tissue damage. TNF is involved in a wide range of immune processes ranging from cell activation and survival to proper organization of lymphoid architecture and immune protection against viruses and intracellular bacteria (112). Additionally however, excess TNF can have severe and deleterious effects in the context of bacteremia-induced septic shock or contributions to tissue destruction in inflammatory diseases such as rheumatoid arthritis and Crohn's disease.

With such a diverse, and potentially damaging set of effector functions, tight regulation of TNF expression is crucial to a healthy immune response. Loss of TNF regulation through the genetic deletion of individual regulatory factors involved can result in infection, auto-immunity, and inflammation (113). Much work has indeed elucidated many layers of transcriptional and post-transcriptional regulation of this pleiotropic cytokine. Transcriptional activation of TNF can involve many factors including NFAT, ATF-2/Jun, and NF-κB (114-116). Post-transcriptionally, both mRNA stability and translation of TNF are known to be highly regulated through the complex interaction of several RNA binding proteins on the TNF 3'UTR (117). The recruitment of these protein complexes is mediated in large part by an AU-rich element (ARE) in the TNF 3'UTR and involves factors that can either promote or inhibit RNA degradation such as

Tristetroprolin (TTP) or HuR (118, 119). Finally, more recent evidence indicates that TNF production and the signaling pathways that induce its expression are also regulated by the activity of endogenous microRNAs (miRNAs) and miRNA machinery. This involves regulation of TLR signaling factors by miR-146a and miR-155 among other miRNAs (120, 121) as well as direct effects on the TNF transcript (122).

Adding further complexity to its function and regulation, TNF is known to be produced by many different immune cell types, including macrophages, T cells, NK cells, and some nonhematopoietic cells as well (112). Although miRNAs play important roles in T cell biology and helper T cells can produce significant amounts of TNF, nearly all of the existing knowledge about miRNA-mediated TNF regulation comes from studies of innate immune cells. We assayed individual miRNAs expressed in T cells for the ability to regulate TNF production and identified specific miRNAs that can influence TNF expression by these cells.

RESULTS

A functional screen for miRNA regulation of TNF production in $CD4^+$ T cells

We and others have shown that miRNA-deficient T cell exhibit dysregulated cytokine production. To address the role of miRNAs in regulating TNF production specifically, we analyzed DGCR8-deficient $CD4^+$ T cells isolated from *Dgcr8*$^{fl/fl}$ *cd4-Cre* mice. In these $CD4^+$ cell cultures, nearly all live cells

produced TNF upon restimulation and this was true for both DGCR8-deficient and wildtype control cells (Figure 1, *top panel*). While there was no difference in the frequency of TNF producing cells, miRNA-deficient cells exhibited a higher mean fluorescent intensity (MFI) for TNF than control cells in co-culture experiments (Figure 1, *bottom panel*). Based on these observations, we reasoned that a screen for miRNA effects on TNF production could be most effectively analyzed using TNF MFI rather than the percentage of positively stained cells. To this extent, we previously established a strategy to screen for miRNA function in helper T cells by introducing individual miRNAs during the in vitro stimulation and culture of miRNA-deficient primary cells. Building upon this strategy, we assayed TNF production by DGCR8-deficient $CD4^+$ cells following transfection with individual miRNAs from a library of 108 miRNAs previously determined to be expressed in mouse $CD4^+$ cells.

The introduction of individual microRNAs into miRNA-deficient $CD4^+$ cells provided a sensitive system for functional screening of TNF. Several miRNAs exhibited a positive effect on TNF production while others repressed TNF production (Figure 2A, Table 1). Most notably, the highest production of TNF was observed in cells transfected with miR-29a and miR-29b (Figure 2A, Table 1). Additionally, miR-155 and miR-146a have been previously implicated as regulators of the TNF production pathway in myeloid cells (120, 121), yet their role in regulating TNF production in $CD4^+$ T cells has not been defined. Our data was consistent with a role for miR-146a in the repression and miR-155 in the promotion of TNF in helper T cells. miR-155 transfection resulted in elevated

TNF production (z = 0.71) and miR-146a resulted in a relative decrease in TNF production (z = -1.09) (Figure 2A). To further validate these findings we carried out additional transfections and observed a consistent and significant increase or decrease in TNF expression by miR-29 and miR-146a, respectively (Figure 2B). Additionally, the co-transfection of miR-29b and miR-146a together resulted in TNF levels nearly identical to that of control-transfected cells (Figure 2C). This finding further demonstrates the opposing effects of miR-146a and miR-29a on TNF production by helper T cells and indicates the importance of combined effects of multiple miRNAs for proper regulation of TNF expression.

We previously demonstrated that miR-29 can repress IFN-γ in a dose dependent manner. Here, a dose-dependent effect of miR-29 on TNF was also observed (Figure 2D, *left panel*). These results were notable for the low concentration of miR-29 that was able to mediate this effect as increased TNF was observed at doses that had little or no effect on IFN-γ (Figure 2D, *right panel*). Finally, an important aspect of TNF biosynthesis is the release of the membrane-bound precursor by the metalloproteinase, TACE (123). To rule out the possibility that the observed increase in TNF staining was due to accumulation of TNF on the cell surface, we stained transfected cells with anti-TNF in the absence of cell-permeabilization. There was a small increase in membrane-bound TNF in miR-29- versus control-transfected cells, but this did not account for the significant increase evident in the intracellular stain (Figure 2C-D). Taken together, these results identify miR-29 as a potent, positive regulator of TNF production in $CD4^+$ T cells.

miR-29 regulation of TNF is T-bet independent

Because miR-29 can directly repress T-bet (see Chapter 2) and T-bet has been shown to inhibit transcription of TNF in myeloid cells (124), we wanted to address possible effects of changes in T-bet, IFN-γ, and Th1 development on TNF production. To do so, and to further validate the results of the initial screen, we carried out an additional screen in *Dgcr8*$^{fl/fl}$ *cd4-cre Tbx21*$^{-/-}$ cells using the same set of miRNAs as before. The deletion of T-bet from miRNA-deficient cells nearly eliminated the IFN-γ production of these cells, but TNF production was not significantly affected (Figure 3A). Importantly, the effect of miR-29 on TNF was independent of T-bet and IFN-γ, as miR-29 significantly increased TNF production in T-bet-deficient cells (Figure 3B, Table 1). Eomes is also a direct target of miR-29, but neither knockdown of Eomes nor combined knockdown of both T-bet and Eomes increased TNF production in helper T cells (Figure 3C). Finally, the results from the TNF screen in T-bet-deficient cells also further validated the findings for miR-155 and miR-146a from the initial screen. miR-146a again decreased TNF expression relative to other miRNAs (z = -1.15) and miR-155 resulted in a significant increase in TNF (z = 2.99) (Figure 3B, Table 1).

miR-29 promotes TNF mRNA expression primarily in a 3'UTR-dependent manner

TNF is regulated at many steps during its production by mechanisms that involve transcription, translation, and mRNA stability. To better understand the

regulation of TNF by miR-29, we assessed the effect of miR-29 on TNF mRNA in both resting and stimulated cells. DGCR8-deficient CD4 T cells were transfected with miR-29 or control miRNA. The following day, a portion of the resting cells were harvested for RNA isolation, and remaining cells were stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin for 4 hours prior to RNA isolation. In resting cells, the introduction of miR-29 appeared to modestly increase TNF mRNA (Figure 4A). Following stimulation, miR-29-transfected cells exhibited a significant increase in TNF mRNA, with approximately two-fold more than in control-transfected cells (Figure 4A). Of note, this difference is roughly equivalent to the increase in TNF protein observed and suggests that the majority of the miR-29 effect on TNF production might be attributable to increased mRNA. Still, these results do not distinguish between changes in transcription versus mRNA stability and do not formally rule out the possibility that miR-29 can also affect TNF translation. To more directly address possible effects of miR-29 on TNF transcription, we co-transfected a TNF promoter luciferase construct with miR-29 or control miRNA in primary T cells. Transfection with miR-29 appeared to increase luciferase expression from the mouse TNF promoter, but this change was variable and did not reach statistical significance when compared to the effect of miR-29 on a control reporter (Figure 4B). These results suggest that miR-29 can increase promoter-dependent transcription of TNF promoter, but that additional mechanisms are likely involved.

Because TNF is tightly regulated through a complex interaction of many post-transcriptional mechanisms, the observed changes in TNF mRNA could be

due to effects on several different post-transcriptional regulatory pathways. To further clarify the mechanism of miR-29 mediated TNF regulation, we utilized luciferase reporter constructs for the Tnf 3'UTR. Just as resting cells transfected with miR-29 exhibited only a small increase in Tnf mRNA, unstimulated cells did not exhibit significantly increased expression of a mouse Tnf 3'UTR reporter (Figure 4C). Following stimulation however, the co-transfection of miR-29 with a full length mouse Tnf 3'UTR reporter consistently resulted in a ~65% increase in relative luciferase expression as compared to co-transfection with a control miRNA (Figure 4C). Similar results were observed using a human Tnf 3'UTR reporter, which exhibited an even greater increase in luciferase expression upon miR-29 co-transfection (Figure 4C). Taken together, these results indicate that miR-29 can mediate its effects on TNF through a conserved region of the Tnf 3'UTR in a stimulation-dependent manner.

Regulation of TNF mRNA by miR-29 depends on the AU-rich element of the TNF 3'UTR

Although miRNAs are well known for their ability to regulate gene expression through direct 3'UTR binding, bioinformatic prediction tools do not identify any candidate sites for direct binding of miR-29 to the Tnf 3'UTR sequence. Importantly however, there are well-characterized and conserved domains of the Tnf 3'UTR known to be involved in the regulation of TNF production. One particularly well-studied domain, the AU-rich element (ARE), can regulate TNF and other cytokine transcripts through protein binding and

recruitment of regulatory complexes (117). Focusing on the ARE as a primary site for recruitment of post-transcriptional regulatory machinery, we utilized a TNF 3'UTR reporter with deletion of the ~70 base-pair ARE (125). Deletion of the ARE did increase reporter expression as compared to the complete TNF 3'UTR (Figure 5A). This was not surprising given the established role of this region in negatively regulating TNF production (126). Notably however, in the absence of the ARE, co-transfection of miR-29 no longer increased reporter expression relative to control transfected cells (Figure 2B). These findings indicate that miR-29 increases TNF production in an ARE-dependent manner.

miR-29 positively regulates TNF production in RAW264.7 cells and $CD8^+$ T cells

While our initial screen was performed in $CD4^+$ T cells, cells of the myeloid lineage are well known to produce large amounts of TNF as a critical component of the innate immune response. As such, we also analyzed macrophages for the effects of altered miR-29 activity on TNF production. miR-29b or miR-29 family inhibitors were transfected into RAW264.7 cells and TNF production was assayed by intracellular staining as before. Even in unstimulated cells, a portion of cells stained positive for intracellular TNF. Notably, miR-29b transfection resulted in an increase in the frequency of this TNF producing population while inhibition of miR-29b decreased the frequency of TNF production (Figure 6A). Following stimulation with LPS, all cells produced TNF and in these conditions miR-29 transfection increased the average TNF production per cell as indicated

by the TNF MFI (Figure 6B-C). Of note, the increase in TNF MFI in RAW264.7 cells (~33%) was less substantial than that observed following miR-29 transfection of DGCR8-deficient T cells (~100%). Perhaps this is in part explained by the fact that miR-29 transfection in RAW264.7 cells represents overexpression of miR-29 rather than ectopic expression in otherwise miRNA-deficient cells. Consistent with the notion of basal miR-29 activity in these cells, inhibition of miR-29 with antisense oligonucleotides decreased TNF production in both unstimulated and LPS-stimulated cells (Figure 6A-C). Taken together, these findings suggest that overexpressed and endogenous miR-29 can function to promote TNF production in myeloid cells as well as $CD4^+$ T cells.

Finally, to further assess the in vivo role of TNF regulation by miR-29 in other cell types, we conducted experiments with overexpression of miR-29 in P14 LCMV-specific TCR transgenic $CD8^+$ T cells. Upon ex vivo restimulation of cells isolated from LCMV infected hosts, we observed a significant increase in the frequency of TNF production among miR-29 overexpressing cells as compared to miR-1 overexpressing controls (Figure 6D). These results demonstrate the ability of miR-29 to influence TNF production by $CD8^+$ cells in response to an in vivo viral infection

DISCUSSION

By introducing individual miRNAs into DGCR8-deficient cells we have revealed a previously unknown function of miR-29 as a positive regulator of TNF

production in helper T cells. Given the ability of miR-29 to repress IFN-γ production (Chapter 2), as well as a recent report describing regulation of the IFN-α receptor by miR-29 (72), the findings described here add to a growing list of immunoregulatory functions for miR-29. It is thus intriguing to consider the evolutionary role of miR-29 as a fine-tuning regulator of pro-inflammatory cytokine signals with the apparent ability to repress at least one arm of inflammation (IFN-γ and IFN-α signaling) while promoting another (TNF expression).

In regards to the regulation TNF, we have shown that miR-29 can increase TNF mRNA and protein and that this effect is mediated in large part through the Tnf 3'UTR. This effect does not appear to involve direct miRNA-3'UTR binding. Instead, our data suggest that miR-29 promotes Tnf mRNA expression by limiting ARE-mediated mRNA destabilization. Although many cytokine transcripts such as IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, and IL-10 are also regulated through 3'UTR AREs, we did not consistently observe a significant increase in the mRNA of these cytokines (data not shown). Many of these genes are not expressed significantly in T cells in our culture conditions, but still, the lack of effect on expressed ARE-containing transcripts such as IL-2 or IL-10 suggests the possibility of ARE-mediated regulation that is specific to TNF and regulated by miR-29. Further understanding the mechanisms of miR-29-mediated regulation of TNF may thus reveal novel factors that contribute specifically to TNF mRNA stability in certain situations.

Of note, tristetraprolin (TTP) is a well-known mediator of post-transcriptional TNF regulation that can be directly repressed by miR-29a in epithelial cells (127). However, in our T cell assays we actually observed an increase in TTP upon miR-29 transfection (data not shown, Chapter 2 microarray data). Thus, the observed effect on TNF in our system is unlikely to result from direct targeting of TTP by miR-29. Instead, our data is consistent with induction of TTP that is secondary to elevated TNF as part of a negative feedback loop involving these two factors (117). Although TTP does not appear to be the causal miR-29 target, our findings suggest that miR-29 can increase TNF expression through repression of one or more of the many factors involved in ARE-mediated decay. Identifying such factors remains an important topic of further investigation.

TNF is clearly an important cytokine capable of mediating protective immunity as well as pathological inflammatory processes. Many cells are capable of producing TNF, yet understanding the functional contributions of TNF production by different cell types remains an active and important area of investigation (128). The data presented here suggest that miR-29 is able to regulate TNF production in $CD4^+$ T cells as well as both $CD8^+$ T cells and myeloid cells. One interesting possibility is that miR-29 can regulate the process of ARE-mediated decay through direct effects on the protein complexes that are recruited to the TNF ARE. Of note however, despite increased TNF protein in RAW 264.7 macrophages following miR-29 expression, TNF 3'UTR luciferase reporters in these cells did not exhibit the same level of increased expression as in helper T cells (data not shown). As different cell-types often differ in their expression

profile for both miRNAs and mRNAs, the notion that a given miRNA may regulate the same functional pathway in different cell types through a different network of target genes remains an intriguing possibility. The increase in TNF protein that we observed in RAW cells may depend less on mRNA stability than the increase observed in T cells. Instead, the effect on TNF in myeloid cells may involve more significant changes in transcription or translation. This is consistent with the fact that we observed some effect of miR-29 on TNF transcription in helper T cells. Clarifying the mechanism of this process might have important implications for understanding transcriptional control of TNF in multiple cell types. Also, T-bet can directly inhibit TNF transcription in RAW cells (124) and we have not formally ruled out the possibility that direct repression of T-bet contributes to increased TNF following miR-29 transfection in these cells. Finally, miR-29 may also indirectly influence the production of TNF during an inflammatory response by altering IFN-α receptor levels (72) and affecting the dynamic interplay of interferon and TNF signaling (112).

The complex regulation of TNF production and the biological relevance of its production by different cell types will continue to be an important area for biomedical research aimed at improving immunotherapies. Integrating miRNAs into our understanding of TNF regulation offers the possibility of revealing new regulatory pathways, new genetic interactions, and new targets for such therapy. Several microRNAs whose expression changes significantly in response to LPS in myeloid cells, including miR-146a, miR-155, and miR-125, have been demonstrated to play an important role in TNF production in these cells. We

report here that several miRNAs, including miR-29 in particular, are capable of exerting significant regulatory effects on TNF production by cells of the adaptive immune system as well. This raises many questions regarding possible feedback loops and coordinated regulation of TNF and miR-29 in T cells. Along these lines, there is strong evidence that NF-κB can act as a transcriptional repressor of the miR-29a/b-1 primary transcript. This suggests one possible model whereby miR-29 might act as a buffer on TNF production as part of a negative feedback loop during an inflammatory response. Specifically, increased NF-κB activity during inflammation could repress miR-29, allowing increased TNF mRNA destabilization and decreased TNF expression. This would not prevent TNF expression, as TNF would continue to be strongly induced through well-described inflammatory signaling pathways, but it could still be an important safeguard on excessive TNF production. Given the inverse effects of miR-29 on TNF and IFN-γ, the functional contribution of miR-29 likely depends on the specific cytokines and cells that are most intimately involved in a given inflammatory response. Understanding cell-type specific mechanisms and physiological settings of miRNA effects on TNF and other cytokines will hopefully help elucidate the complex cross-talk of inflammatory signals that underlies a healthy immune response.

EXPERIMENTAL PROCEDURES

Mice

Dgcr8$^{fl/fl}$, CD4-Cre, Rosa26-Stop-loxP-YFP, Rosa26-Stop-loxp-tdRFP, and *Tbet$^{-/-}$* mice have been described previously (80, 81, 129, 130). All mice were housed and bred in specific pathogen-free conditions in the Animal Barrier Facility at the University of California, San Francisco. All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of California, San Francisco.

Cell stimulation and culture

$CD4^+$ T cells from spleen and lymph nodes of young mice (4-7 weeks old) were isolated by magnetic bead selection (Dynal). Purified $CD4^+$ T cells were stimulated with hamster anti-mouse CD3 (clone 2C11, 0.25 µg/mL) and anti-mouse CD28 (clone 37.51, 1 µg/mL) on plates coated with goat anti-hamster IgG (0.3 mg/mL in PBS for 2 hours at room temperature; MP Biomedicals) for 60-68 hours at an initial cell density of 0.7-1 x 10^6 cells/mL. Following stimulation, cells were expanded in media with 20 units/mL of recombinant IL-2 (National Cancer Institute). The resulting cultures were free of $CD8^+$ T cells (<1%) when analyzed by flow cytometry 5 d after activation. For experiments involving carboxyfluorescein succinimidy ester (CFSE), cells were labeled for eight minutes with 5 uM CFSE, quenched with an equal volume of fetal bovine serum (FBS), and washed two times in 10% FBS prior to stimulation and culture. All T cell culture was in DMEM high glucose media supplemented with 10% FBS, pyruvate, nonessential amino acids, MEM vitamins, L-arginine, L-asparagine, L-

glutamine, folic acid, beta mercaptoethanol, penicillin, and streptomycin. RAW264.7 cells were cultured in 10 cm plates in DMEM supplemented with 10% FBS pyruvate, nonessential amino acids, MEM vitamins, L-arginine, L-asparagine, L-glutamine, folic acid, beta mercaptoethanol, penicillin, and streptomycin. Cells were passaged every three days and approximately 10^6 cells were used to seed a fresh plate.

Transfection and miRNA oligonucleotides.

Cells were transfected using the Neon electroporation transfection system (Invitrogen) with an optimized version of the manufacturers recommended protocol. Briefly, transfections were performed using 2-3 x 10^7 cells/mL in 10 µL "T" buffer (Invitrogen) with a miRNA concentration of 500 nM unless otherwise specified. Optimized Neon transfection system setting was 1550 V with three 10 ms pulses. All transfection experiments involving cytokine analysis consisted of one transfection after 24 hours of stimulation and a second transfection after 90-100 hours of total culture time. For the initial transfection, cells were removed from plates, transfected, and returned to fresh plate-bound stimulation (anti-CD3, anti-CD28). Following the second transfection, cells were returned to media with 20 units/mL IL-2. miRIDIAN miRNA mimics were from Dharmacon. Transfections of RAW264.7 cells were performed using 2-3 x 10^7 cells/mL in 10 µL "R" buffer (Invitrogen) with a miRNA concentration of 500 nM and three 10 ms pulses at 1650 V. For inhibition of miR-29, miR-29a and miR-29b antisense hairpin inhibitors (Dharmacon) were combined to a final concentration of 500 nM of each

for transfection. Negative control miRNA mimic and inhibitor were based on cel-miR-67 (mature sequence UCACAACCUCCUAGAAAGAGUAGA, Dharmacon).

miRNA screening and analysis

For miRNA screens, cells were stimulated at 1 x 10^6 cells/mL for 24 hour in 6-well plates. Cells were removed from stimulation, pooled, and 0.2 x 10^6 cells were transfected with each individual miRNA. Following transfection, cells were placed in separate goat anti-hamster IgG-coated wells of a 24-well plate in 0.5 mL media with fresh anti-CD3 and anti-CD28. Availability of mice and cells allowed 16-24 miRNAs to be screened at one time such that the complete screen of all miRNAs consisted of 5 batches. CFSE analysis by flow cytometry on day 4 was used to calculate a proliferation index. Proliferation index is the average number of cell divisions of cells that underwent at least one division (Flowjo analysis software). 0.2 x 10^6 cells of the remaining cells from each well were transfected again on day 4 and restimulation for intracellular staining was performed on day 5. Within each batch of the complete screen, the proliferation index and the frequency of TNF producing cells was normalized to the batch median to give a TNF score. These values were then used to generate *Z* scores for the entire set of miRNAs ($Z = x\text{-mean/SD}$, where x is the TNF score for each individual miRNA).

Intracellular staining and antibodies

For intracellular cytokine analysis, T cells were restimulated for four hours with 10 nM phorbol 12-myristate 13-acetate (PMA) and 1 μM ionomycin in the presence of 5 μg/mL brefeldin A (BFA) to allow intracellular cytokine

accumulation. RAW 264.7 cells were stimulated with 0.1 ng/mL or 1 ng/mL lipopolysaccharide (LPS) in the presence of 5 µg/mL BFA. For all cytokine stains, cells were fixed with 4% formaldahyde and subsequently permeabilized and stained in PBS containing 0.5% saponin, 1% bovine serum albumin, and 0.1% sodium azide. Fluorophore-conjugated antibodies including Percp-e710-TNF, eFluro450-IFN-γ, allophycocyanin (APC)-IL-4 were from eBioscience. Stained cells were analyzed with a LSRII and FACSDiva software (BD Biosciences) as well as Flowjo analysis software.

RNA isolation and qPCR

Total RNA was isolated with Trizol LS reagent (Invitrogen). For mRNA expression analysis cells were harvested on day 5, 24 hours after miRNA transfection.Total RNA was oligo(dT)-primed for first strand cDNA synthesis (Superscript III Kit, Invitrogen). Quantitative real-time fluorogenic 5′-nuclease PCR were performed with Solaris mRNA assays (Dharmacon) for *Tnf* according to the manufacturers instructions. Gapdh sense 5'-CTCGTCCCGTAGACAAAATGG-3'; Gapdh antisense 5'-AATCTCCACTTTGCCACTGCA-3'; Gapdh probe FAM-5'-CGGATTTGGCCGTATTGGGCG-3'.

Luciferase Reporter assays

The 3'UTR or promoter of TNF was amplified from murine activated T cell cDNA (full-length mouse 3'UTR and mouse TNF promoter), SV40-LUC-3'-UTR-ARE plasmid provided by V. Kruys (ARE-deficient mouse 3'UTR), or human T cell cDNA (human full length 3'UTR). 3'UTR PCR products were cloned into the

psiCHECK-2 dual luciferase reporter construct (Promega) downstream of renilla luciferase between XhoI and NotI restriction sites. Promoter PCR products were cloned into pGL3 between XhoI and BglII. $CD4^+$ T cells were stimulated and expanded *in vitro* and co-transfected on day 4 of culture with reporter constructs containing the 3'UTR of interest along with miRNA or miRNA inhibitor oligonucleotides. For promoter reporter assays, pGL3 reporter was co-transfected with TK renilla for normalization along with miRNA. Luciferase activity was measured 24 after transfection using the Dual Luciferase Reporter Assay System (Promega) and a FLUOstar Optima plate-reader (BMG Labtech). Primers used to amplify the 3'UTRs included:

mouse TNF UTR fwd, 5'-CTCGAGGGGAATGGGTGTTCATCC -3'

Mouse TNF UTR rev, 5'-GCGGCCGCTTTATTTCTCTCAATGACCCG -3';

human TNF UTR fwd, 5'-CTCGAGGGAGGACGAACATCCAAC-3';

human TNF UTR rev, 5'-GCGGCCGCTTTCTTTTCTAAGCAAAC-3'.

Mouse TNF promoter fwd, 5'-TCGAGCAGCTTAACTGCCGGAGGAG-3';

mouse TNF promoter rev, 5'-AGATCTGCTTCTGCTGGCTGGCTG-3'.

Transduction, adoptive transfer, and LCMV infection

$CD8^+$ cells were activated *in vivo*, transduced, and transferred as described (105) and splenocytes were analyzed on d7 post-transfer. Cells were stimulated with 0.2 uM gp33 peptide for 5 hours followed by intracellular cytokine staining. Pri-miR constructs were generated by amplifying and subcloning the endogenous miRNA hairpin with ~150 nucleotides of 5' and 3' flanking sequence into MSCV-IRES-Thy1.1 DEST. Pri-miR-29a parent construct was provided by A.Goga.

FIGURES AND TABLES

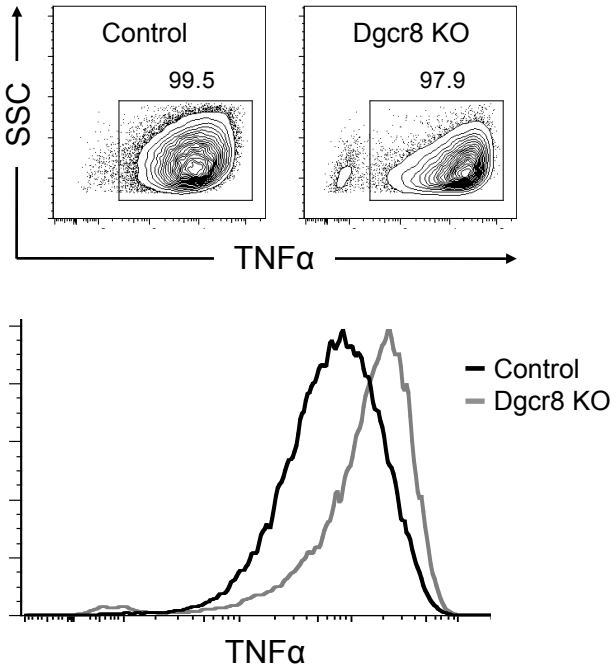

Figure 1.

Increased TNF production by DGCR8-deficient CD4$^+$ T cells

TNF production of co-cultured CD4$^+$ T cells isolated from *Dgcr8$^{fl/fl}$ Cd4-cre R26-tdRFP* and *Dgcr8$^{+/fl}$ Cd4-cre R26-YFP* mice. Cells were co-cultured for 5 days and RFP and YFP fluorescence were used to distinguish *Dgcr8$^{fl/fl}$* and *Dgcr8$^{+/fl}$* cells, respectively, at time of analysis. All viability dye-excluding cells are shown. Data are representative of 3 independent experiments.

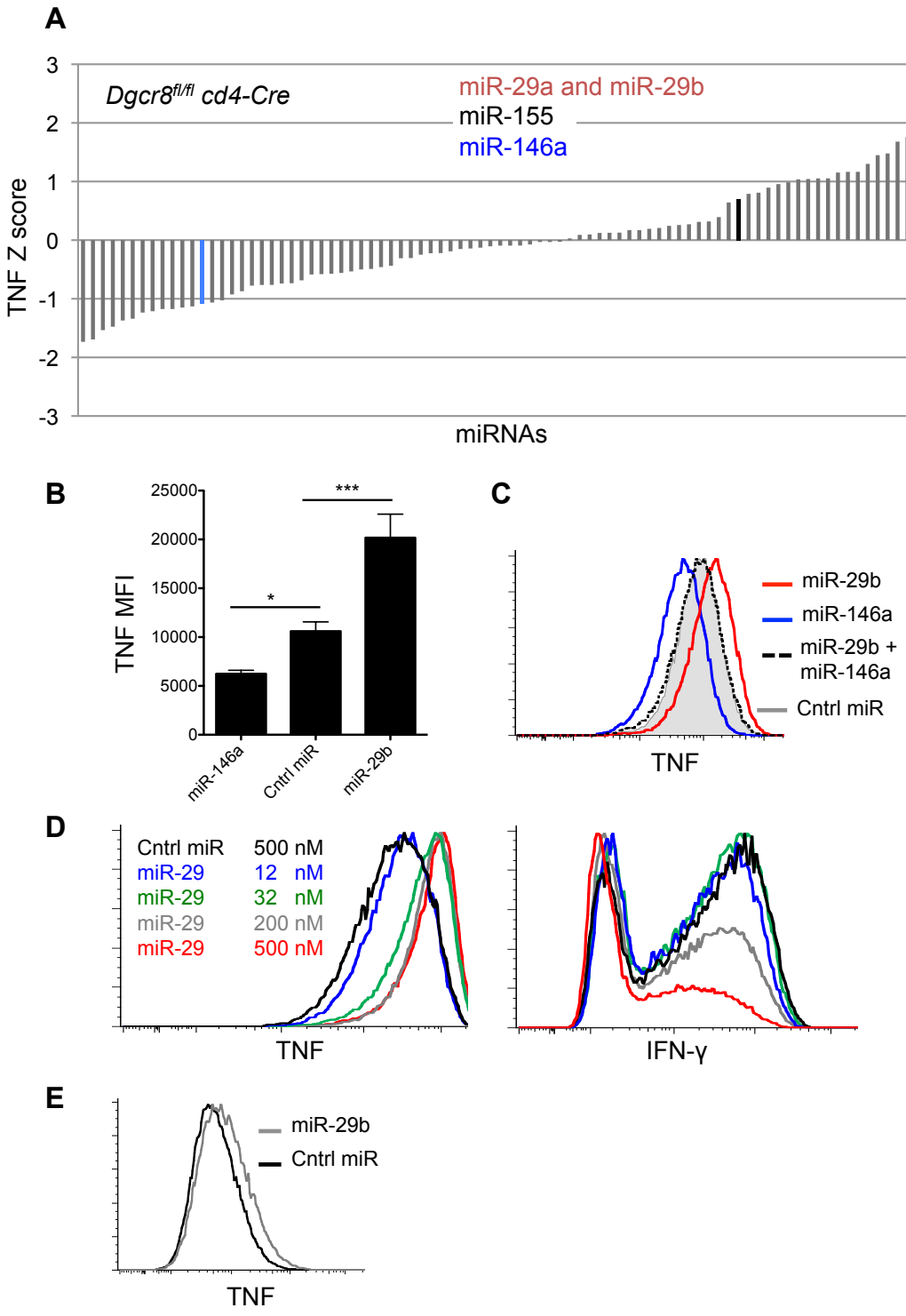

Figure 2.

MiR-29a and miR-29b increase TNF production by CD4$^+$ T cells stimulated in vitro

(A) *Z* scores for mean fluorescence intensity (MFI) of TNF staining among restimulated DGCR8-deficient cells transfected with individual miRNAs. miRNAs with a proliferation score of $Z < -1.5$ or $Z > 1.5$ were not included in the TNF analysis due to possible indirect effects of survival or proliferation on cytokine production.

(B-C) DGCR8-deficient cells were transfected with miR-146a, control miRNA, or miR-29b as indicated. Values are average MFIs ± SD from three independent transfections and representative of two independent experiments, $*p<0.05$, $**p<0.01$; ANOVA Tukey's post-hoc test.

(D) Intracellular cytokine stains for TNF (left panel) and IFN-γ (right) among *Dgcr8*$^{fl/fl}$ *cd4-Cre* CD4^{+} T cells transfected with increasing concentrations of miR-29b

(E) Membrane-bound TNF stain. miR-29b- and control-transfected cells were stained with anti-TNF in the absence of permeabilization by saponin at time of staining.

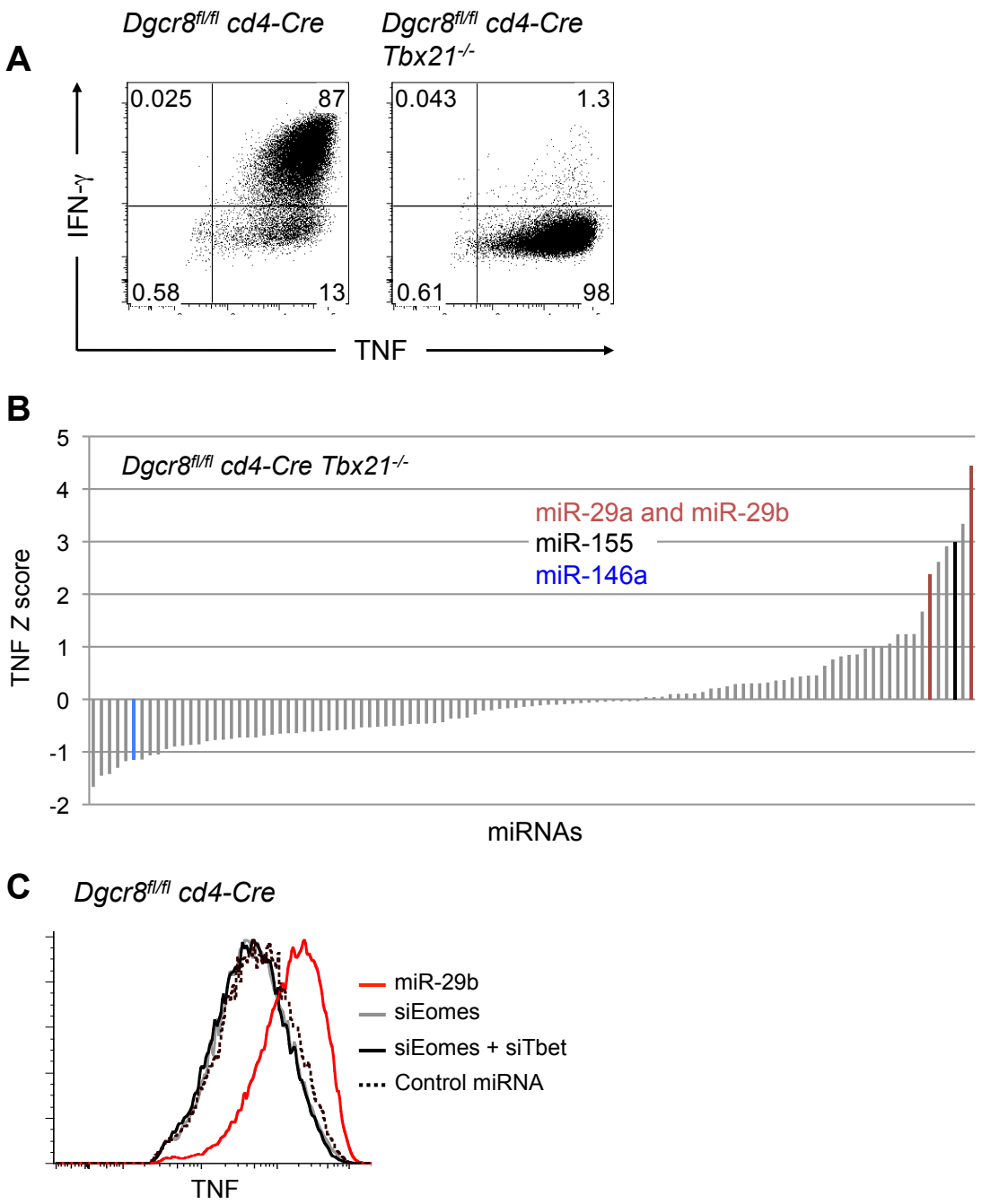

Figure 3.

MiR-29a and miR-29b induced increase in TNF production by CD4$^+$ T cells is independent of T-bet

(A) Cytokine production of CD4$^+$ T cells isolated from *Dgcr8$^{fl/fl}$ Cd4-cre* and *Dgcr8$^{fl/fl}$ Cd4-cre Tbx21$^{-/-}$* mice.

(B) *Z* scores for mean fluorescence intensity (MFI) of TNF staining among restimulated DGCR8-deficient, *Tbx21*$^{-/-}$ cells transfected with individual miRNAs. (C) DGCR8-deficient cells were transfected with miR-29b or siRNA targeting T-bet (siTbet) and/or Eomes (siEomes) as indicated and stained for intracellular TNF. Data is representative of two independent experiments.

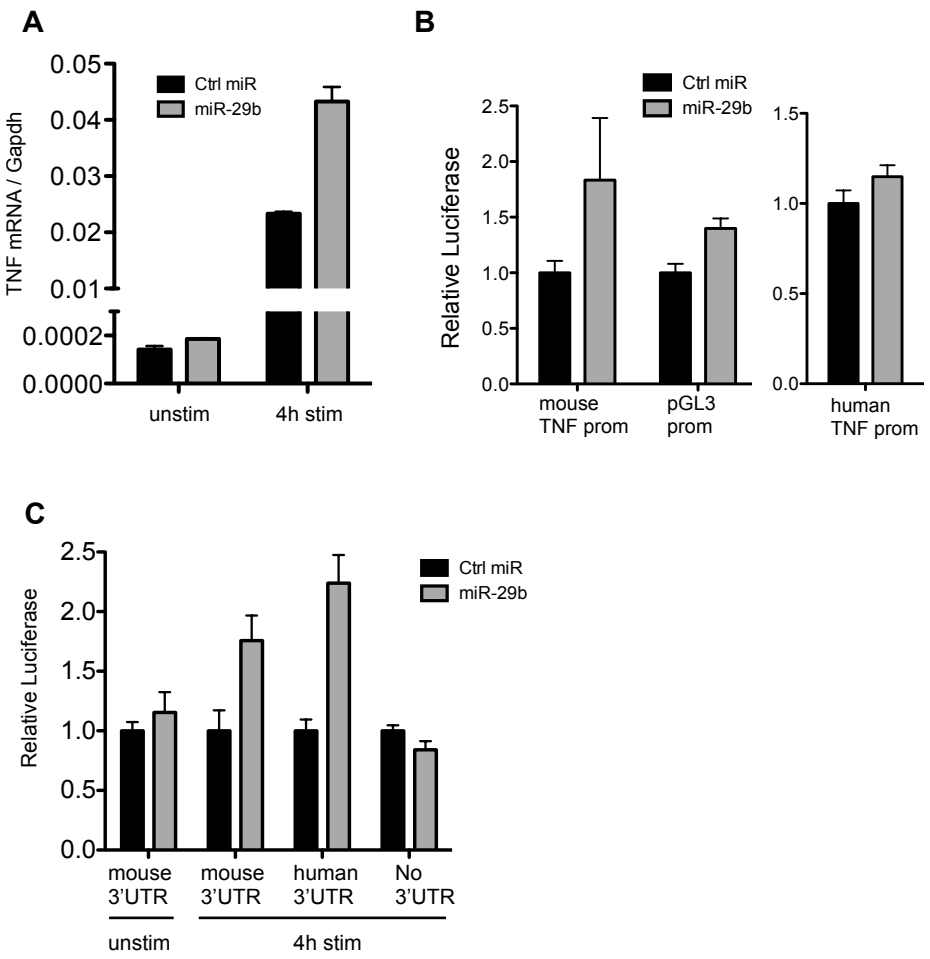

Figure 4.

miR-29 promotes Tnf mRNA expression primarily in a 3'UTR-dependent manner

(A) Real-time qPCR analysis of *Tnf* mRNA expression 24 hours after DGCR8-deficient cells were transfected with miR-29b or control miRNA. Cells were either unstimulated in complete media or stimulated for 4 hours with PMA and Ionomycin in complete media before being harvested for RNA. mRNA was normalized to *Gapdh*. Values are average ± SD from three independent transfections.

(B-C) Primary *Dgcr8*$^{fl/fl}$ *Cd4-cre* CD4 T cells were co-transfected with a firefly TNF promoter luciferase vector and a renilla control vector (B) or a dual luciferase reporter (C) along with miR-29b or control miRNA. Luciferase reporters contained ~1000 bp of the proximal *Tnf* promoter from mouse or human (B) or the full-length mouse or human *Tnf* 3'UTR (C). Cells were stimulated for four hours with PMA and ionomycin unless otherwise noted. Reporter luciferase activity was measured 24 hours after transfection and normalized to control luciferase. All values are relative to normalized luciferase activity in control transfected cells. Data are representative of three independent experiments and values are means ± SD from three independent transfections.

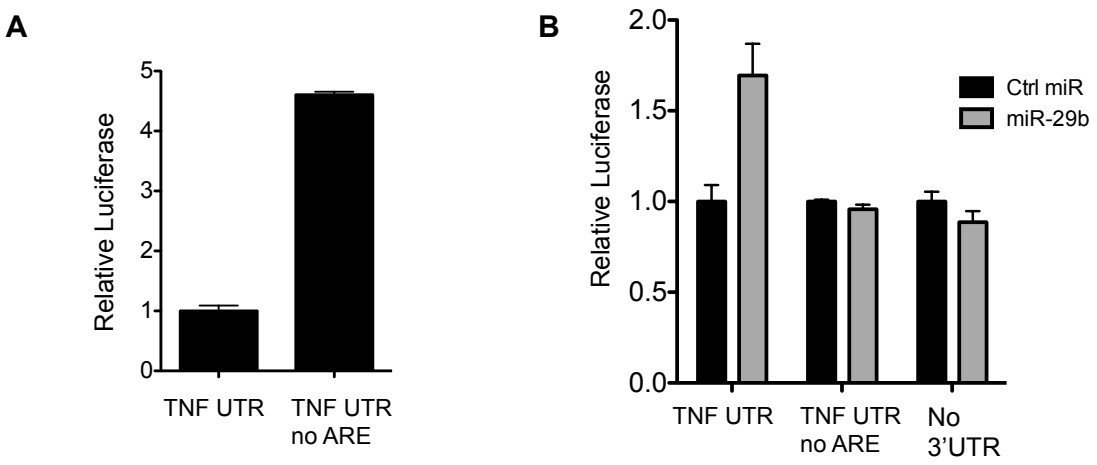

Figure 5.

Regulation of TNF mRNA by miR-29 depends on the AU-rich element of the TNF 3'UTR

(A-B) Primary *Dgcr8*$^{fl/fl}$ *Cd4-cre* CD4 T cells were transfected with a dual luciferase reporter (A) or co-transfected with a dual luciferase reporter and miR-29b or control miRNA (B). Luciferase reporters contained the full-length mouse 3'UTR of *Tnf* (TNF UTR) or the mouse 3'UTR of *Tnf* lacking the 70 base-pair AU-rich element (no ARE). Cells were stimulated for 4 hours. Renilla luciferase activity was measured 24 hours after transfection and normalized to firefly luciferase activity. Values are relative to normalized luciferase activity in control-transfected cells. Data are representative of three independent experiments and values are means ± SD from three independent transfections.

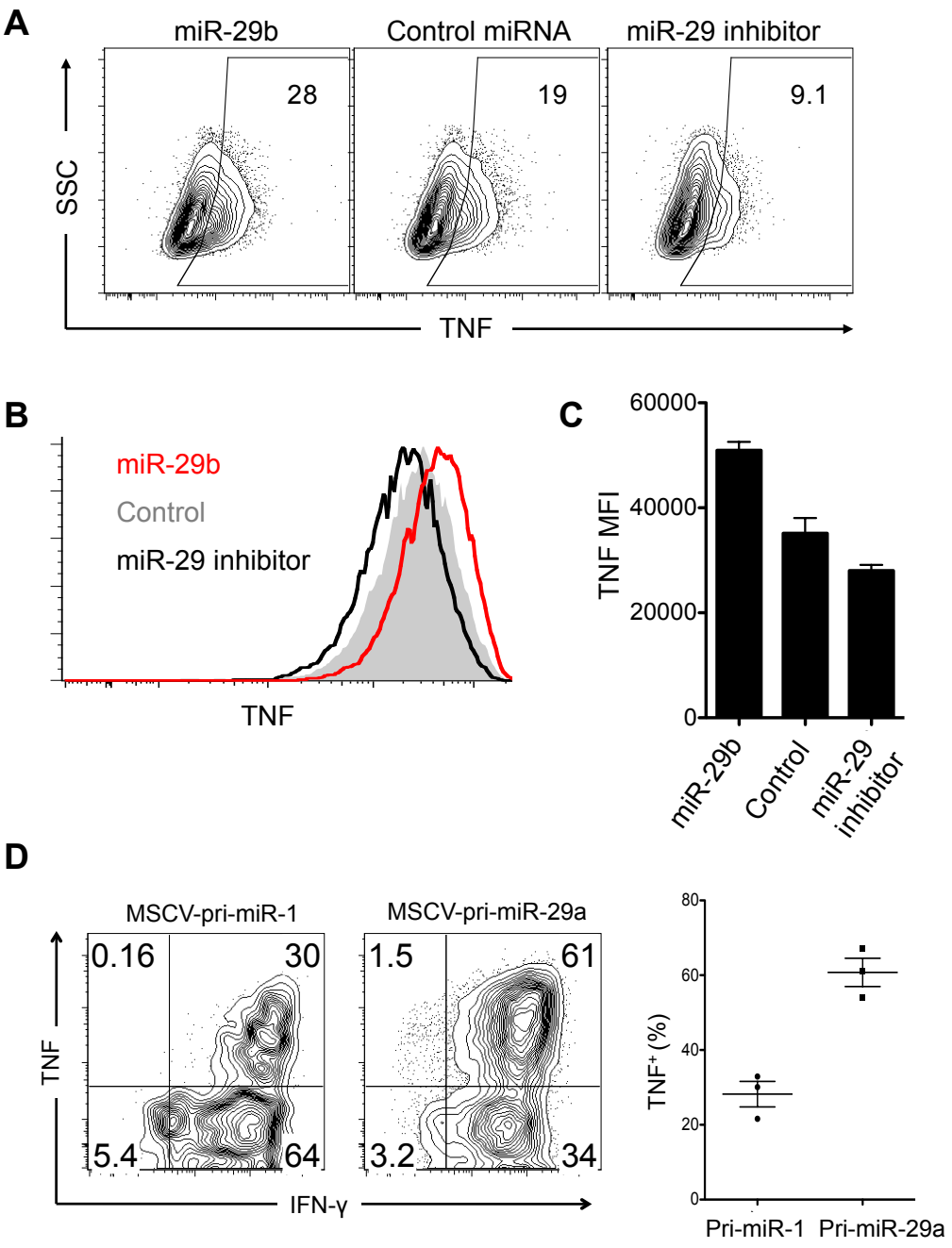

Figure 6.

MiR-29 postively regulates TNF production in additional cell types

(A-C) RAW 264.7 cells were transfected with miR-29b, control miRNA, or miR-29 family inhibitors and analyzed 24 hours following transfection. Cells were

incubated with Brefeldin A for 4 hours (A) or stimulated for 4 hours in 1 ng/mL LPS with Brefeldin A (B-C) prior to intracellular TNF staining. Values are average ± range of two indpendent transfections (C) and data is repesentative of three independent experiments.

(D) Frequency of TNF production among miRNA-overexpressing cells following transduction and adoptive transfer of P14 cells during LCMV infection. Transferred and transduced cells were identified by congenic CD45.1 and retroviral Thy1.1 expression, respectively; n=3, data is representative of two independent experiments.

Table 1. Summary of *Z* scores for TNF MFI following miRNA transfection

Dgcr8 KO		Dgcr8 T-bet double KO	
miRNA	**TNF *Z* score**	**miRNA**	**TNF *Z* score**
mmu-miR-181c	-1.735	mmu-miR-467c	-1.663
mmu-miR-467c	-1.694	mmu-miR-467a	-1.453
mmu-miR-140	-1.535	mmu-miR-181b	-1.422
mmu-miR-181b	-1.477	mmu-miR-467e	-1.302
mmu-miR-7a	-1.371	mmu-miR-7a	-1.176
mmu-miR-24	-1.340	mmu-miR-146a	-1.146
mmu-miR-467a	-1.236	mmu-miR-130b	-1.145
mmu-miR-130b	-1.213	mmu-miR-101b	-1.066
mmu-miR-151-3p	-1.176	mmu-miR-205	-1.049
mmu-miR-669c	-1.175	mmu-miR-181a	-0.946
mmu-miR-96	-1.150	mmu-miR-25	-0.897
mmu-miR-148a	-1.131	mmu-miR-363	-0.882
mmu-miR-146a	-1.087	mmu-miR-212	-0.867
mmu-miR-101b	-1.064	mmu-miR-17	-0.857
mmu-miR-26a	-0.926	mmu-miR-32	-0.798
mmu-miR-328	-0.872	mmu-miR-24	-0.774
mmu-miR-674	-0.776	mmu-miR-151-3p	-0.769
mmu-miR-16	-0.765	mmu-miR-185	-0.752
mmu-miR-19b	-0.764	mmu-miR-301a	-0.727
mmu-miR-144	-0.740	mmu-miR-466g	-0.727
mmu-miR-22	-0.735	mmu-miR-148a	-0.690
mmu-miR-363	-0.685	mmu-miR-186	-0.674
mmu-miR-423-3p	-0.586	mmu-let-7b	-0.653
mmu-miR-205	-0.580	mmu-miR-423-5p	-0.645
mmu-miR-301a	-0.573	mmu-miR-652	-0.644
mmu-miR-106b	-0.560	mmu-miR-181c	-0.617
mmu-miR-342-5p	-0.534	mmu-miR-140	-0.616
mmu-miR-183	-0.500	mmu-miR-30e	-0.606
mmu-miR-421	-0.492	mmu-miR-26b	-0.594
mmu-miR-26b	-0.437	mmu-miR-298	-0.587
mmu-miR-20a	-0.309	mmu-miR-674	-0.576
mmu-miR-19a	-0.306	mmu-miR-125	-0.570
mmu-miR-31	-0.248	mmu-miR-148b	-0.534
mmu-miR-193	-0.224	mmu-miR-18a	-0.533
mmu-miR-652	-0.221	mmu-miR-183	-0.523
mmu-miR-744	-0.191	mmu-miR-296-5p	-0.515
mmu-miR-33	-0.152	mmu-miR-500	-0.503
mmu-let-7f	-0.143	mmu-miR-466l	-0.502
mmu-let-7b	-0.129	mmu-miR-342-3p	-0.471
mmu-miR-30c	-0.106	mmu-miR-22	-0.466

mmu-miR-181a	-0.094
mmu-miR-466l	-0.093
mmu-miR-326	-0.092
mmu-miR-27a	-0.073
mmu-let-7i	-0.020
mmu-miR-378	-0.011
mmu-miR-182	0.003
mmu-miR-532	0.029
mmu-miR-126-3p	0.090
mmu-miR-340-5p	0.098
mmu-miR-20b	0.123
mmu-miR-466g	0.126
mmu-miR-194	0.128
mmu-let-7g	0.170
mmu-miR-21	0.171
mmu-miR-342-3p	0.193
mmu-miR-186	0.208
mmu-miR-18a	0.244
mmu-miR-500	0.258
mmu-miR-142-3p	0.270
mmu-let-7c	0.312
mmu-miR-141	0.319
mmu-miR-297a	0.393
mmu-miR-296-5p	0.642
mmu-miR-155	0.704
mmu-miR-142-5p	0.789
mmu-miR-200c	0.809
mmu-miR-374	0.896
mmu-miR-375	0.956
mmu-miR-361	0.987
mmu-miR-720	1.043
mmu-miR-872	1.053
mmu-miR-27b	1.054
mmu-miR-185	1.153
mmu-miR-9	1.165
mmu-miR-92a	1.165
mmu-miR-191	1.301
mmu-miR-467e	1.449
mmu-miR-30e	1.479
mmu-miR-30b	1.680
mmu-miR-30d	1.758
mmu-miR-98	1.779
mmu-let-7d	2.602
mmu-miR-29a	2.653
mmu-miR-29b	2.690

mmu-miR-326	-0.463
mmu-miR-141	-0.453
mmu-miR-132	-0.436
mmu-miR-21	-0.365
mmu-miR-744	-0.364
mmu-miR-93	-0.347
mmu-miR-106b	-0.289
mmu-miR-342-5p	-0.216
mmu-miR-16	-0.209
mmu-miR-872	-0.180
mmu-miR-142-3p	-0.170
mmu-miR-26a	-0.163
mmu-let-7a	-0.142
mmu-miR-103	-0.131
mmu-miR-194	-0.116
mmu-let-7f	-0.103
mmu-miR-106a	-0.103
mmu-miR-144	-0.093
mmu-miR-142-5p	-0.085
mmu-let-7i	-0.072
mmu-miR-669c	-0.064
mmu-let-7c	-0.053
mmu-miR-15a	-0.045
mmu-miR-375	-0.034
mmu-miR-191	-0.025
mmu-miR-92a	0.000
mmu-miR-340-5p	0.003
mmu-miR-700	0.042
mmu-miR-19a	0.042
mmu-miR-200c	0.050
mmu-miR-98	0.099
mmu-let-7g	0.106
mmu-miR-421	0.109
mmu-miR-19b	0.112
mmu-miR-20b	0.140
mmu-miR-30d	0.205
mmu-miR-532-5p	0.215
mmu-miR-31	0.248
mmu-miR-23a	0.291
mmu-miR-33	0.300
mmu-miR-9	0.305
mmu-miR-126-3p	0.305
mmu-miR-378	0.321
mmu-miR-20a	0.358
mmu-miR-128	0.366
mmu-miR-15b	0.415

mmu-miR-328	0.437
mmu-miR-210	0.451
mmu-miR-361	0.456
mmu-miR-96	0.639
mmu-miR-423-3p	0.759
mmu-miR-28	0.817
mmu-miR-296-3p	0.847
mmu-miR-720	0.854
mmu-miR-27a	0.965
mmu-miR-425	1.000
mmu-miR-147	1.009
mmu-miR-27b	1.059
mmu-miR-297a	1.236
mmu-miR-193	1.239
mmu-let-7d	1.242
mmu-miR-182	1.669
mmu-miR-29a	2.379
mmu-miR-374	2.616
mmu-miR-30b	2.913
mmu-miR-155	2.993
mmu-miR-30c	3.339
mmu-miR-29b	4.446

CHAPTER 4:

CONCLUSIONS, DISCUSSION, AND FUTURE DIRECTIONS

The integration of miRNAs into our working knowledge of helper T cell biology adds another layer to the complex network of gene expression that controls the function of these cells. MicroRNAs are distinct in their ability to regulate target genes in a unique temporal manner that does not require new protein synthesis and in a qualitative manner notable for the “fine-tuning” potential of modest or incomplete target repression. We are only beginning to understand how and when these distinctive properties of miRNAs can influence the expression of signaling molecules, transcription factors, and epigenetic modifications that underlie helper T cell differentiation and cytokine production. Adding to the complexity of miRNA biology, not only do miRNAs uniquely regulate networks of target genes, but miRNAs themselves appear to be highly regulated in the context of functionally significant feedback loops. This involves transcriptional control of individual pri-miRs as well as changes in miRNA-processing machinery that can further influence miRNA activity. Even the susceptibility of target genes to miRNA activity in T cells can be a regulated factor through activation-induced changes in 3’UTR length (131). Understanding miRNA target pathways in the context of regulated changes in miRNA activity will offer valuable insight into the ultimate biological relevance of miRNA-mediated changes in gene expression.

One miRNA, many targets, multiple functions

A fundamental characteristic of miRNAs is the ability of a single miRNA to directly regulate hundreds of genes in a given cell type. Further, the complete set of actively regulated miRNA-target genes can depend on the gene expression programs specific to that cell. These properties give an individual miRNA the potential to have distinct effects on different cell lineages, a capacity exemplified by miR-29 through its independent effects on helper T cell cytokine production, thymic epithelial cell responsiveness (72), leukemia (104), and stromal cell mediated fibrosis (65). At the same time, a large set of target genes implies that miRNA studies are oversimplified in describing only one target gene that is responsible for an observed phenotype. Indeed, our work demonstrates that a single miR-29 target was insufficient to fully account for the observed functional effects of this miRNA. But even the identification of two functional homologues as relevant targets leaves a considerable amount to be learned about the role of miR-29 in helper T cells, let alone other cell types. Still, this work is consistent with the intriguing notion that miRNAs have evolved to regulate networks of genes that are part of identical or overlapping functional programs rather than single genes. The combined efforts of bioinformaticists and molecular immunologists to understand these networks not only has the potential to clarify mechanisms of miRNA function, but also to discover previously unrecognized gene interactions. While the field has advanced substantially by identifying individual target-function relationships of specific miRNAs, the incorporation of

expanded networks of miRNA-targets into our understanding of miRNA function will be an important goal for future work.

In regards to the notion that a single miRNA regulates several genes in the same functional pathway, a recent study reports IFN-γ itself as a direct target of miR-29 (62). In combination with our results, this finding suggests the simultaneous regulation of Eomes, T-bet, and IFN-γ by miR-29 and further demonstrates the impressive potential for a single miRNA to target functionally significant nodes of gene expression. In our own study however, despite the presence of a conserved miR-29 binding site in the 3'UTR of IFN-γ, we did not observe repression of an IFN-γ 3'UTR reporter by miR-29 in helper T cells. This may be due in part to the effects of other 3'UTR elements that dominate the post-transcriptional regulation of IFN-γ in T cells (100). Another intriguing possibility consistent with these findings is that by evolving to target multiple genes in the same functional pathway, miR-29 is able to influence IFN-γ production in different cell types and/or different physiological situations through unique target combinations. Ultimately, understanding the dynamic functional contribution of specific targets may depend on the in vivo introduction of target genes with mutated miR-29 binding sites and in the context of different immune challenges.

The diverse of effects of a single miRNA can be compounded even further in instances where target genes themselves have distinct functions in different cell types. As it relates to the work presented here, T-bet and Eomes are intriguing functional targets of miR-29 due to their multi-faceted roles in multiple immune lineages. For example, while T-bet is critical for normal IFN-γ production

and Th1 differentiation in CD4 T cells, it is dispensable for IFN-γ production by CD8 T cells (130). This is likely due in part to high expression of a functional homologue, Eomes, in CD8 cells but not CD4 cells (76). Eomes is still not the whole story, however, for natural killer cells are largely dependent on T-bet for their cytokine-induced IFN-γ production despite high Eomes expression (130). The complex and context-dependent roles of these two miR-29 targets in CD8 cells is further illustrated by the simultaneously redundant and reciprocal effects of T-bet and Eomes on effector and central memory CD8 cell differentiation (83, 93, 132). Our finding that miR-29 can simultaneously regulate these two transcription factors likely has important implications for other immune cell lineages, including CD8 T cells, that depend on proper regulation of T-bet and Eomes for their differentiation and function. Future work will hopefully help elucidate the significance of miR-29-mediated effects on T-bet, Eomes, and the many other targets of this miRNA in a variety of cell lineages.

Making sense of microRNA expression in Th1 and Th2 cells

Based on the significantly different gene expression programs of helper T cell subsets, one might have reasonably hypothesized that differentially expressed miRNAs could contribute to the induction and maintenance of distinct helper T cell lineages. One interesting observation regarding the role of miRNAs in helper T cell differentiation, however, is that very few miRNAs are differentially expressed between Th1 and Th2 cells (49, 50). Still, a lack of extensive miRNA differences between Th1 and Th2 cells is not necessarily inconsistent with the

ability of miRNAs to differentially regulate important aspects of distinct T cell lineages. One simple possibility is that current reports of miRNA expression among helper T cell subsets are too limited in their timing and that there may in fact be lineage-specific differences in miRNA expression at important and specific time points not yet analyzed. However, more biologically interesting explanations can also be proposed. For example, an activated T cell receives a combination of many signals during the differentiation process. The cell must integrate this complex set of inputs to generate, for the most part, a digital output in the form of a differentiation program. By regulating the expression of key genes in the positive feedback loops of helper T cell differentiation, miRNAs may act as a buffer to prevent weak signals from leading to the inappropriate differentiation of one lineage. In this way, similar miRNA expression among different lineages could still ensure that a T cell adopts a given effector fate only in the presence of adequate polarizing stimuli.

The regulation of T-bet and Eomes by miR-29 may be a useful example of this type of buffering in certain situations. In such a model, miR-29 could keep modest Th1-inducing signals in check and allow for sufficient stimulation and induction of Th2 differentiation (Figure 1, *top*). The signals for appropriate Th1-differentiation, on the other hand, are presumably strong enough to overcome this miR-29-mediated regulation (Figure 1, *bottom*). In the absence of miR-29 however, early elevation of T-bet and Eomes could induce inappropriate differentiation into IFN-γ producing cells despite ongoing Th2 polarizing signals (Figure 1, *middle*). Additionally, the quantitative effects of miRNAs on gene

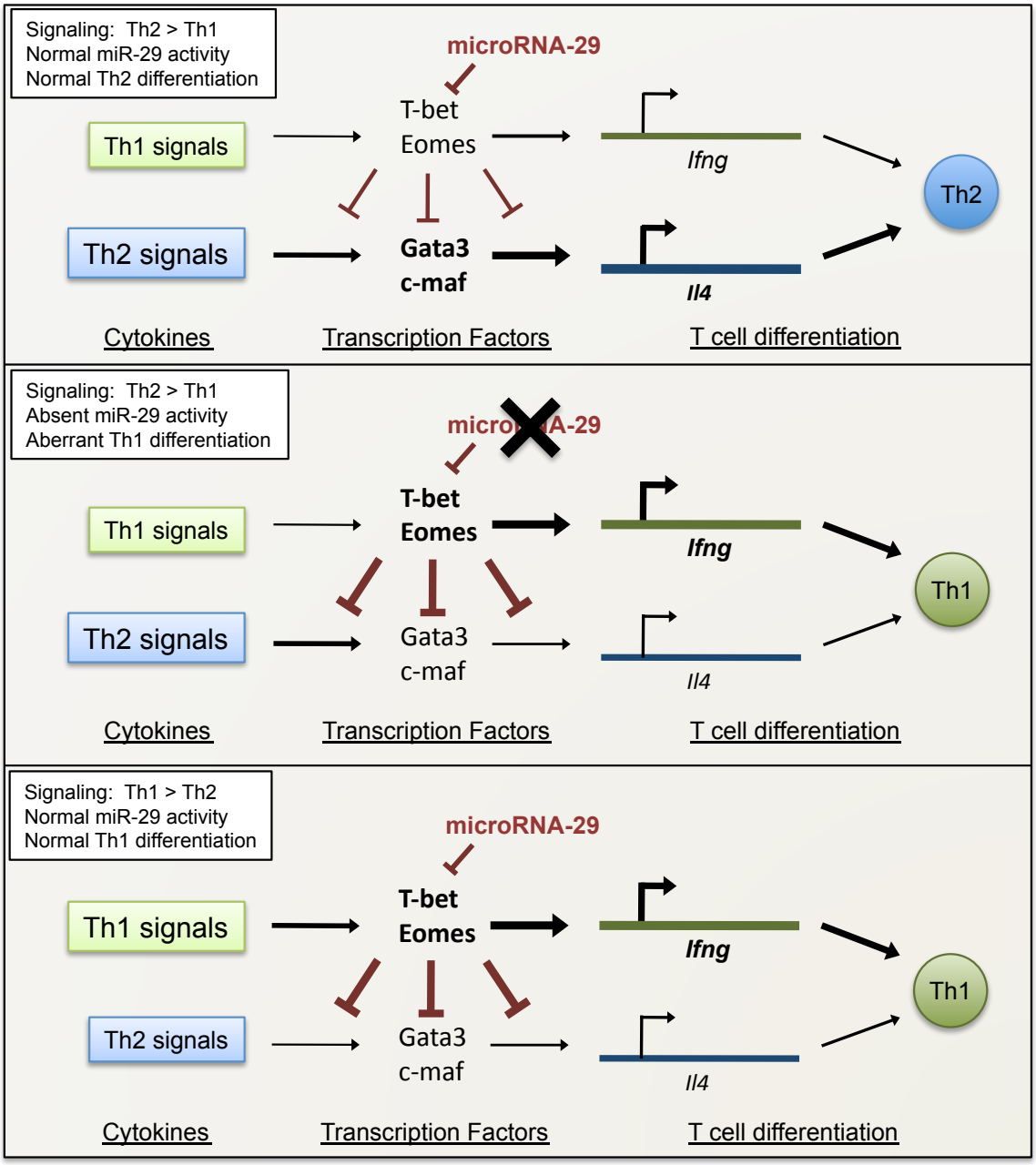

Figure 1. Model of miR-29 activity as a buffer of Th1-inducing signals

expression can be directly dependent on the relative abundance of target gene to miRNA. Specifically, a target gene can be significantly repressed when below an expression threshold, but minimally repressed when above that threshold (133). This notion may be especially important in the induction and maintenance of helper T cell differentiation programs as these programs involve sensitive feedback loops and likely require maintained repression of lineage-specific genes in a threshold-sensitive manner. In this way, the networks of miRNA-mediated gene regulation in helper T cells could differ between lineages due to differences in transcript abundance despite similar miRNA expression profiles.

Thinking about therapeutics

As our knowledge of miRNA function in the immune system has rapidly progressed in the last several years, so has the interest in making use of miRNA function for therapeutic purposes. The function of individual miRNAs including miR-155, miR-146a, and now miR-29b in modulating the inflammatory response of helper T cells has implicated these regulatory molecules as interesting therapeutic targets for treatment of pathological inflammatory processes. The direct targeting of T-bet by miR-29 is particularly intriguing given the established role of this transcription factor in many mouse models of autoimmunity as well as its increased expression in subsets of patients with multiple sclerosis (134, 135). Furthermore, $CD4^+$ T cells of asthma patients exhibit decreased T-bet expression (136) and specific stimulation of T-bet has been proposed as a possible therapeutic strategy for asthma (95). Our results indicate that modulating miR-29

activity could have exciting therapeutic potential based on its ability to regulate T-bet and IFN-γ production, yet such an approach is certainly not without its own challenges and caveats. While the design and synthesis of miRNA mimics and inhibitors are relatively straightforward as far as small molecule therapeutics are concerned, the targeted delivery of these molecules to relevant cells remains an impediment to their clinical value. Advancements in nanoparticle technology and exosome biology currently represent promising areas for improved miRNA delivery (137, 138), but use of these tools in the lab, let alone the clinic, is still in its infancy. Even if the obstacle of delivery is overcome, our results suggest that independent from repression of T-bet and IFN-γ, miR-29 can also unexpectedly promote TNF production. Although more work needs to be done to understand the mechanism and reach of this finding, based on the complex pro-inflammatory potential of TNF, this effect should be considered carefully if evaluating miR-29-based therapeutics.

Finding Functional Targets

A successful, unbiased approach to identifying miR-29 targets that influence TNF and IFN-γ expression has the exciting potential to reveal novel gene interactions of inflammatory pathways. However, the most straightforward approaches for experimental validation of miRNA targets, such as expression analysis and 3'UTR luciferase assays, require a specific and focused list of candidate genes that are most readily selected based on a known function in the pathway of interest. Although miRNA target prediction programs exist that might

allow a more unbiased approach, these algorithms predict hundreds to thousands of possible targets for any given miRNA and these lists inevitably include functionally irrelevant genes, genes that are not expressed in the cell type of interest, and false predictions (139). As one intriguing alternative, a global gene knockdown screen to identify genes whose knockdown phenocopies miRNA activity has proven effective in at least one case (140). However, this requires the tools and resources necessary to carry out a large-scale functional screen in the relevant cell type.

To overcome some of these challenges, we utilized an approach that draws upon elements of both target prediction and gene knockdown screens by first combining miR-29 target predictions with our own experimental data from miR-29 gain of function and loss of function studies to produce a more focused list of candidate targets. Notably, this approach combined data from 1) the especially sensitive system of individual miRNA expression in otherwise miRNA-deficient cells, 2) experimentally observed changes in gene expression following inhibition of endogenous miRNA, and 3) an available target prediction algorithm designed to minimize false positive predictions. By implementing this strategy in the same experimental setting as an observed miRNA function, we believe this approach can generate a set of candidate targets most relevant to the cell type and phenotype of interest. Indeed, both T-bet and Eomes were identified through this analysis and subsequently validated as functionally relevant targets of miR-29 and its effects on IFN-γ. We further utilized this list of candidate miR-29 targets to carry out a knockdown screen of these ~100 genes with the goal of

finding a gene whose knockdown would result in an increase in TNF expression similar to that observed upon miR-29 transfection. Several siRNAs initially appeared to phenocopy the miR-29 effect on TNF, but knockdown of these same genes with additional siRNAs failed to validate these findings. Although this small siRNA screen has not identified an individual miR-29 target that is dominantly responsible for increased TNF expression, it remains possible that repression of some combination of these genes is ultimately necessary for this effect. Just as combined knockdown of T-bet and Eomes was necessary to recapitulate the effects of miR-29 on IFN-γ, a combination of siRNAs with modest individual effects on TNF may offer additional insight into the regulation of this important cytokine. Our efforts to clarify the targets that contribute to miR-29-mediated changes in both IFN-γ and TNF expression illustrate some of the challenges of miRNA-target identification as well as the potential value of continued advancements in both experimental and bioinformatic approaches.

In Conclusion

The work presented here utilizes and describes a useful strategy for identifying miRNA function in primary cells and demonstrates a significant contribution of at least one miRNA family, miR-29, in regulating cytokine production pathways of helper T cells. This work is significant as one of few studies to date to describe a pathway of helper T cell effector function regulated by a single miRNA, but it is still just an early step forward in this field. Much as we have just recently begun to appreciate the interaction, cooperativity, and

antagonism of various transcription factors in immune cell function, our understanding of miRNAs in the immune system will benefit significantly from advancements in studying the combined, cooperative, and opposing effects of different miRNAs. Hopefully future work will expand on our findings to elucidate the regulatory significance of 1) individual miRNAs on large networks of gene expression, 2) the cooperative effects of several miRNAs on these networks, and 3) the functional consequences of changes in the relative expression of miRNAs and their targets. Understanding miRNA-mediated regulation in immune cells has the exciting potential to reveal novel functional overlap among genes and to better inform constantly evolving maps of gene interaction networks. In this way, continued efforts in the study of miRNAs and immunity have important implications for developing immune-targeted therapies and shaping our understanding of the immune response in health and disease.

APPENDIX

Section 1: Identifying miRNAs that can regulate IL-4 and IL-13 production

Introduction

The differentiation of naïve helper T cells into Th2 cells results in IL-4, IL-5, and IL-13 producing effector cells. These cytokines have important implications for the inflammatory processes that underlie allergy and asthma including cytokine mediated recruitment of eosinophils and mast cells, stimulation of mucus production by epithelial cells, and IgE antibody production. Whether an activated T cell differentiates into a Th2 cell depends on several factors, among which, IL-4 signaling through STAT6 to promote GATA3 expression represents a critical component. Because IL-4 production can further reinforce its own expression in both an autocrine and paracrine manner, a strong positive feed back loop of Th2 inflammation can be readily established. To maintain healthy immunity, careful regulation of the Th2 differentiation program involves many components, from signaling events to transcription factor activation to epigenetic changes in cytokine loci themselves.

Due to the autoregulatory nature of IL-4 production, small changes in regulated processes can lead to significant changes in IL-4 production in vitro and in vivo (74). Particularly relevant to the work presented here, one notable example of this sensitive system is exhibited by microRNA-155- (miR-155-) deficient helper T cells, which exhibit a marked Th2 differentiation bias despite

the relatively modest regulatory effects of a single miRNA on an indivdiual target gene (43, 44). We hypothesized that additional miRNAs also contribute to the proper regulation of Th2 differentiation and carried a functional screen for miRNA effects on IL-4 in a manner similar to that described in chapters 2 and 3 for IFN-γ and TNFα, respectively.

Results

Cells lacking both DGCR8 and T-bet provide a sensitive system to screen for miRNA regulation of IL-4 production

As described in previous chapters, our general screening strategy involved transfection of individual miRNAs into miRNA-deficient cells followed by intracellular cytokine staining of re-stimulated cells. Notably however, one challenge to utilizing this approach to assay IL-4 production is that miRNA-deficient cells exhibit increased T-bet expression and an overwhelming propensity to produce IFN-γ that might confound the regulatory processes of normal Th2 differentiation (see Chapter 2). To address this issue, we established mice with T cells deficient for both the miRNA biogenesis factor, DGCR8, as well as the Th1-inducing transcription factor, T-bet. Notably, when cultured in Th1 polarizing conditions, these miRNA-deficient, T-bet-deficient cells were more likely to produce both IFN-γ and IL-4 than cells deficient for T-bet alone (Figure 1). This supports the notion that miRNAs can restrain cytokine production and demonstrates T-bet independent regulation of cytokine production by miRNAs. In

non-polarizing conditions, very few of these DGCR8/T-bet 'double knockout' cells produced IFN-γ, but they readily produced IL-4 when cultured and re-stimulated in vitro (Figure 1). Taken together, these results indicate that DGCR8-, T-bet-deficient cells can provide a useful system to screen for miRNA-mediated regulation of IL-4 production following miRNA transfection.

Individual miRNAs regulate IL-4 production by helper T cells

We screened a library of 108 miRNAs and found that several were independently capable of increasing or decreasing the frequency of IL-4 production by helper T cells (Figure 2, Table 1). Most notably, miR-19a and miR-19b as well as miR-130b and miR-301a (which share a seed sequence that differs from that of miR-19 by just one nucelotide) significantly increased IL-4 production (Figure 2). Several seed-family pairs also decreased IL-4 production, including miR-27a and miR-27b, miR-26a and miR-26b, and miR-132 and miR-212 (Figure 2). miR-24 and miR-140-5p also markedly decreased IL-4 production, although these findings were not strengthened by the initial support of a "seed-family replicate" in the same screen (Figure 2). To further validate these findings, we carried out additional transfection experiments with only those miRNAs that exhibited the most significant increase or decrease in IL-4 production ($z > 2$ or $z < -1$, respectively) (Figure 2B). Two notable exceptions that did not appear consistent with the initial screen were let-7a, which did not repress IL-4 in validation experiments, and miR-700. Although miR-700 was notable for the highest IL-4 and IL-13 production in the initial screen (Figure 2), its effects

were not nearly as substantial in follow-up experiments. For the most part however, the initial findings were validated and the “IL-4-promoting miRNAs” (Figure 2B, *gray bars*) were clearly distinguishable from the “IL-4-repressing miRNAs” (Figure 2B, *black bars*). Additionally, several technical replicates of control miRNA-transfected samples exhibited intermediate IL-4 production (Figure 2B, *green bars*). The range of IL-4 production observed following the transfection of unique miRNAs was also impressive, ranging from fewer than 10% IL-4^+ cells on the low end to greater than 50% on the high end. The four independent control miRNA transfections averaged 17.1 ± 3.4% (Figure 2B).

The miRNAs of the miR-23/24/27 cluster can cooperatively repress IL-4 production

Because miR-23a, -24-2, and -27a are expressed together from a single primary transcript, it was intriguing that each was able to independently reduce the frequency of IL-4 producing cells. As miRNAs can function in a cooperative manner and these miRNAs are expressed together in the same miRNA cluster, we cotransfected all three miRNAs together to address the possibility of cooperative regulatory effects on IL-4 production. Notably, the effect of all three miRNAs together was greater than the effect of each miRNA individually, even when the total concentration of transfected miRNA(s) was equivalent (Figure 2B, *leftmost bar*) (For combined transfection, the concentration of each individual miRNA was 170 nM and the total miRNA concentration was ~500 nM.) This finding suggests that the miR-23/24/27 cluster represents an important node of

regulation in IL-4 production and that transcriptional regulation of this miRNA cluster and the cooperative nature of these miRNAs have important implications for Th2 differentiation.

Specific microRNAs exhibit independent effects on IL-4 and IL-13

Because IL-13 is produced by Th2 cells and contributes meaningfully to the immune function of these cells, we also analyzed IL-13 production in our initial screen. The production of IL-4 and IL-13 was highly correlated suggesting that, for the most part, there was little independent regulation of these two cytokines mediated by miRNAs (Figure 3A). A few notable exceptions however, involved the let-7 family, miR-24, and miR-155. Cells transfected with let-7 family miRNAs appeared to produce less IL-13 than expected based on the relative IL-13 to IL-4 production trend observed for the other miRNAs (Figure 3A, *red markers*). This trend was perhaps modest, but was reinforced by the existence of multiple data points corresponding to the several let-7 family members in the data set. This finding is also consistent with a published role for let-7 miRNAs in the specfic repression of IL-13 (141). Similarly, despite higher than average IL-4 production in miR-155-transfected cells, these cells did not exhibit elevated IL-13 production. This observation is intriguing given the Th2 bias of miR-155-deficient cells, but at the present time has not been followed up any further.

Individual transfection of miR-24 consistently reduced IL-4 production but did not appear to reduce the frequency of IL-13 production to a similar extent. This was especially apparent when miR-24 effects on IL-13 production were

analyzed only with other miRNAs found to repress IL-4 (Figure 3B, *blue marker*). This finding suggests that miR-24 may be able to specifically repress components of the IL-4 production pathway that are independent from IL-13 expression. The mechanism for this unique regulation remains to be determined and offers the potential to reveal additional layers of Th2 differentiation. Of note, when miR-24 was co-transfected with miR-23a and miR-27a, both IL-13 and IL-4 were markedly repressed (Figure 3B, *orange marker*). Taken together, these findings regarding IL-13 highlight the sensitive and effective nature of this screen for identifying individual miRNAs that can regulate Th2 cell differentiation and cytokine production in helper T cells.

MicroRNA regulation of Th1 and Th2 differentiation in wildtype cells

To better understand the potential functional significance of those miRNAs with effects on Th2 cell cytokine production, we also transfected wildtype cells with individual miRNAs. Granted, such miRNA transfection in wildtype cells represents 'overexpression' of a given miRNA and thus might be expected to have limited effects due to the potentially saturating activity of endogenous miRNA. However, the miRNA-deficient, T-bet KO cells used for the initial screen do not undergo normal Th1 differentiation and thus cannot directly reveal miRNA-mediated influences on the physiological balance between IL-4-producing Th2 cells and IFN-γ-producing Th1 cells. Wildtype cells, on the other hand, provide a system to allow analysis of this possibility. Following miRNA transfection in wildtype cells, individual miRNAs did result in different amounts of IL-4

production and we observed a highly anti-correlated relationship between IFN-γ and IL-4 production as expected (Figure 4). Most notably, miR-27a, miR-27b, and miR-140-5p decreased the frequency of IL-4 production and increased the frequency of IFN-γ production in wildtype cells (Figure 4). Also similar to the findings in miRNA-deficient cells, miR-19 resulted in a relative increase in IL-4 producing cells. These findings are consistent with the mutually antagonistic nature of Th1 and Th2 cell gene expression programs and further support the notion that changes in miRNA activity can have significant consequences helper T cell differentiation.

Conclusions and Discussion

As with the miRNA functional screens of the previous chapters, the initial conclusions in the IL-4 screen described here were strengthened for miRNAs with seed-matched family member miRNAs that also had similar effects. In this case, both miR-19a and miR-19b increased IL-4 production while miR-26a and 26b, miR-27a and miR-27b, and miR-132 and miR-212 all decreased IL-4 production. These findings further validate the sensitive nature of this screening strategy for reproducibly identifying functional capabilities of individual miRNAs. Additionally, follow up experiments validated the IL-4 repression observed by miR-140-5p and miR-24, two miRNAs without an additional seed family member in the library of screened miRNAs. Of note, miR-140-5p is actually the lower expressed mature miRNA of the miR-140 RNA duplex while miR-140-3p is more

highly expressed in helper T cells. In one study, miR-140-3p was even reported as the third most highly expressed miRNA in helper T cells, only behind let-7 and miR-92 (50). As such, if mechanisms emerge for the regulated selection of different mature miRNAs from the same pre-miR, miR-140 could be a particularly important substrate for such regulation. Because the pri-miR-140 transcript must be abundant in order to maintain the high expression of miR-140-3p, a small shift towards the preferential RISC loading of miR-140-5p instead could have significant consequences on miR-140-5p targets and Th2 differentiation.

The finding that miR-23, miR-24, and miR-27 are apparently able to cooperatively repress IL-4 production is another intriguing finding of this work. These three miRNAs are expressed as part of the same pri-miR transcript, and there is indeed a precedent from the miR-17-92 cluster for miRNAs expressed together to act cooperatively on both unique and overlapping targets to regulate cell function. Determining whether these three miRNAs are regulating the same genes in a synergistic manner and/or a network of different genes in the same functional pathway is an important future direction of this work. Answering these questions has the potential to reveal new genes in the Th2 differentiation pathway and to better elucidate the complex, cooperative capabilities of miRNA activity. Further, the increased functional effect of co-transfecting several miRNAs raises the intriguing if not challenging possibility of carrying out additional functional screens with combinations of miRNAs. This could involve miRNAs grouped by genomic organization, expression patterns, predicted target sets, or even unbiased combinations. Currently, important limiting factors for

such experiments involve the cost of the miRNA mimics themselves and the throughput of primary T cell transfection.

The observation that miR-19a and miR-19b increased IL-4 production implicates a new role for this well-studied miRNA. miR-19 is strongly induced upon cell activation as part of the miR-17~92 cluster and is perhaps best known for its ability to regulate cell survival through direct effects on PTEN (51, 52). Recently, miR-19 has also been implicated in the promotion of IFN-γ production (55) and we also observed a relative increase in IFN-γ production following miR-19 (see Chapter 2). Given the mutually antagonistic features of Th1 and Th2 differentiation, the apparent capability of miR-19 to promote both IFN-γ and IL-4 production seems contradictory. Perhaps however, the effective function of miR-19 depends on the current pattern of gene expression and the present state of T cell differentiation. In this way, miR-19 may not necessarily determine the differentiation fate of an activated T cell, but could reinforce an initiated program both by promoting survival of the activated cell and by contributing to the regulation of a gene network that supports effector cytokine producing potential. The integration of this highly expressed and highly regulated miRNA into the IL-4 production pathway implies a complex interaction of miR-19's ability to regulate both cell survival and cytokine production as part of the activation induced changes in helper T cell gene expression. Future work to elucidate the mechanisms that underlie the observations described here will further integrate miRNAs into our knowledge of helper T cell differentiation.

Figures and Table

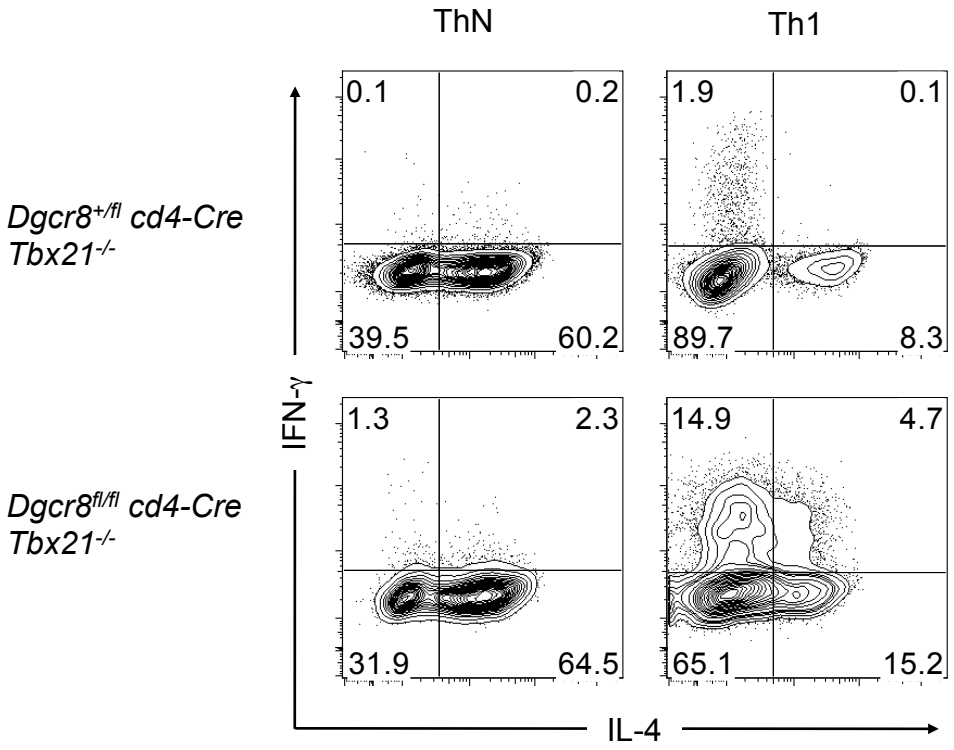

Figure 1.

Cytokine production by helper T cells lacking both T-bet and miRNAs

$CD4^+$ cells were isolated from mice of the indicated genotypes and stimulated in non-polarizing (ThN) or Th1 polarizing conditions (10 ng/mL IL-12, 10 ug/mL anti-IL-4).

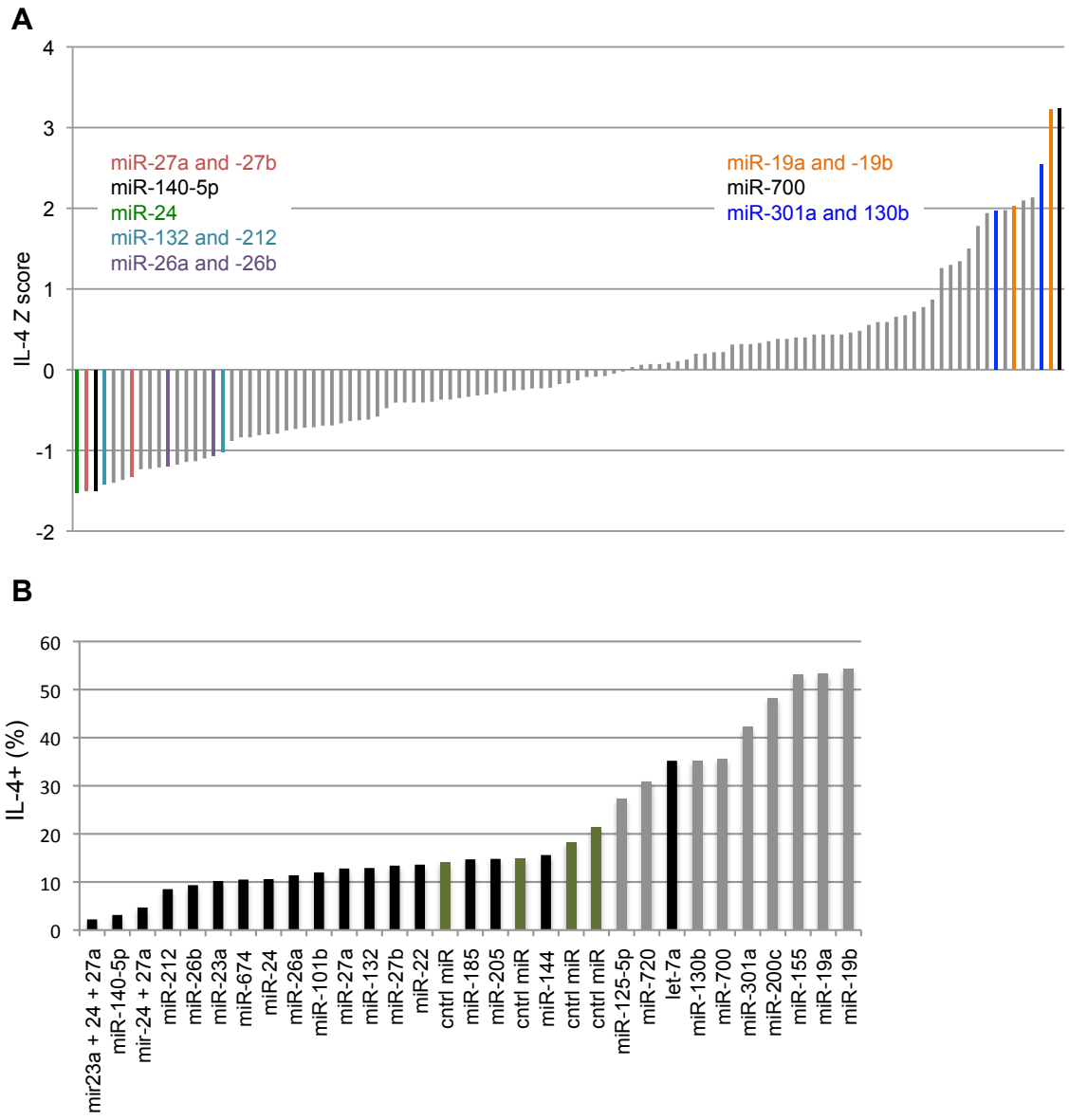

Figure 2.

miRNAs regulate IL-4 production in DGCR8- and T-bet-deficient helper T cells.

(A) *Z* scores for frequency of IL-4 production among restimulated DGCR8-, T-bet-deficient cells transfected with individual miRNAs. MiRNAs with a

proliferation score of $Z < -1.5$ or $Z > 1.5$ were not included in the IFN-γ analysis due to possible indirect effects of survival or proliferation on cytokine production. (B) miRNAs that most significantly increased or repressed IL-4 production in (A) ($z > 2$ or $z < -1$, respectively) were selected for additional transfection and cytokine assays. Black bars are miRNAs that reduced IL-4 in initial screen; gray bars are miRNAs that increased IL-4 in initial screen; green bars are technical replicates of a control miRNA. Total concentration of miRNA for transfection in all samples was 500 nM.

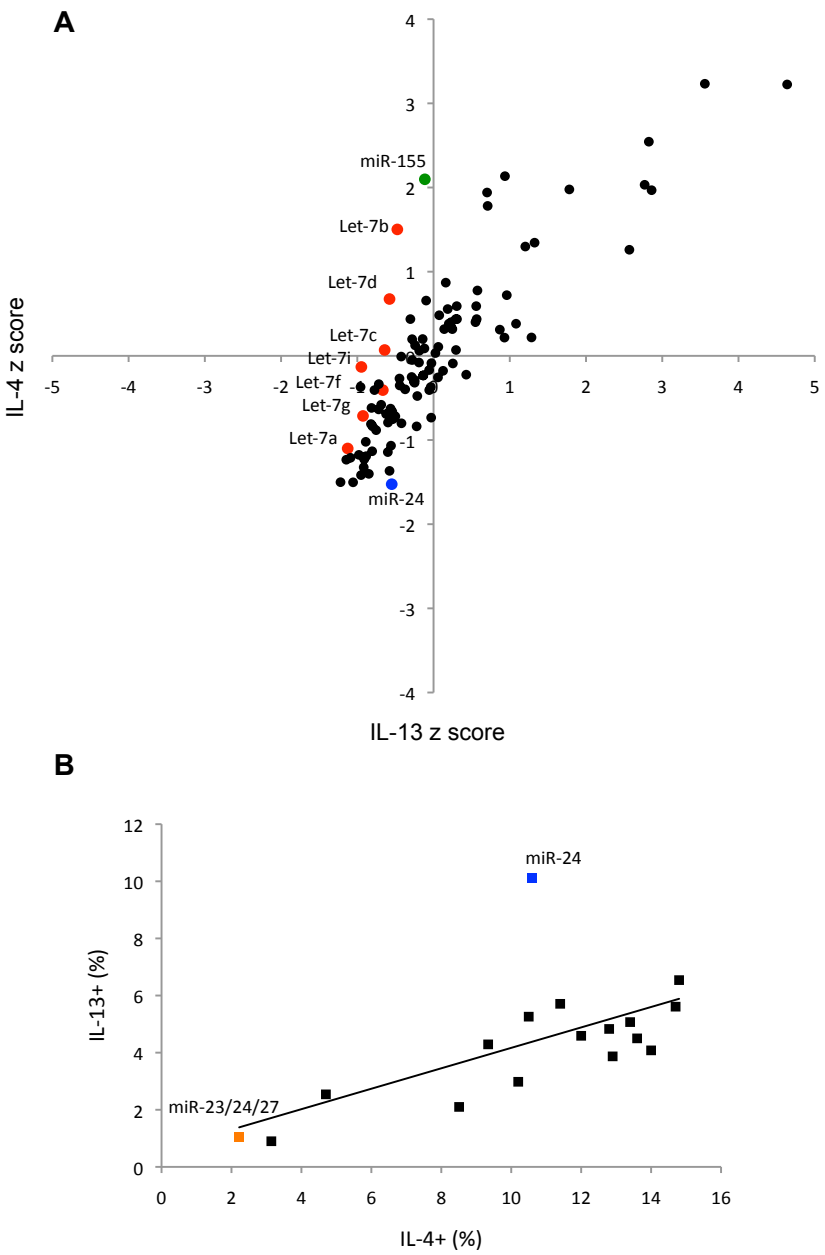

Figure 3.

Specific microRNAs exhibit independent effects on IL-4 and IL-13

A) IL-4 and IL-13 expression for all miRNAs screened. Notable outliers highlighted as indicated.

B) IL-4 and IL-13 expression for miRNAs that repress IL-4. Data is from same validation experiment as shown in Figure 2B.

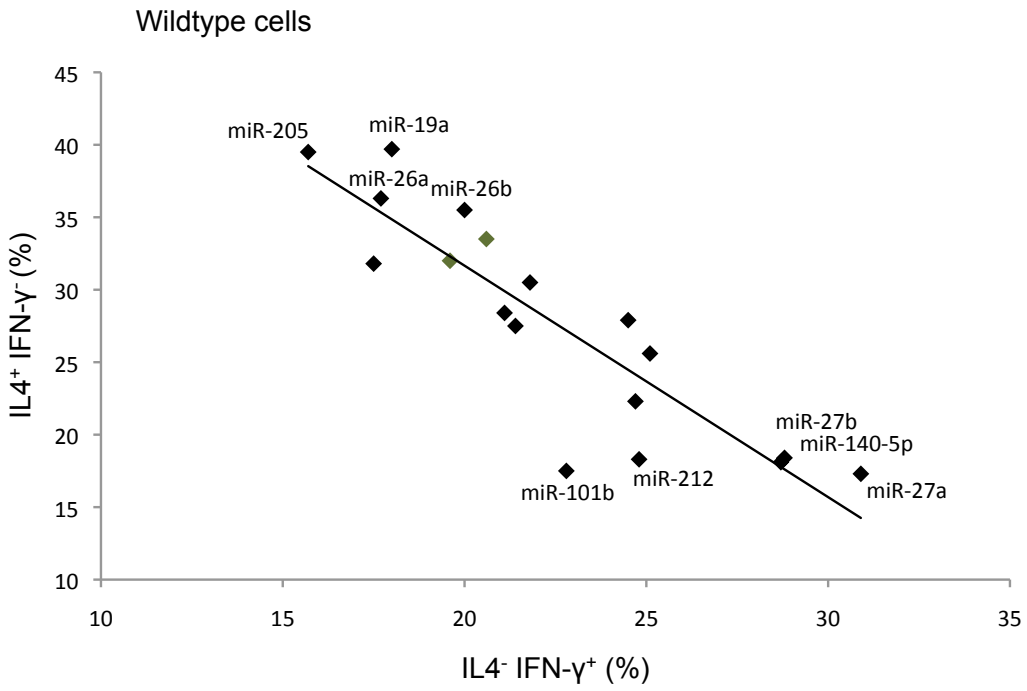

Figure 4.

Effect of individual miRNAs on Th1 vs. Th2 differentiation in wildtype helper T cells

Wildtype CD4 cells were transfected with individual miRNAs that were selected based on their ability to significantly regulate IL-4 production in miRNA-deficient, T-bet-deficient cells. Green markers represent control miRNA-transfected samples.

Table 1. Summary of *Z* scores for IL-4 production following miRNA transfection in CD4 T cells deficient for both T-bet and DGCR8.

microRNA	IL-4 *Z* score
mmu-miR-24	-1.525
mmu-miR-27b	-1.503
mmu-miR-140-5p	-1.501
mmu-miR-132	-1.417
mmu-miR-185	-1.402
mmu-miR-22	-1.367
mmu-miR-27a	-1.324
mmu-miR-101b	-1.234
mmu-miR-144	-1.229
mmu-miR-17	-1.211
mmu-miR-26a	-1.192
mmu-miR-205	-1.176
mmu-miR-29b	-1.143
mmu-miR-674	-1.133
mmu-let-7a	-1.100
mmu-miR-26b	-1.067
mmu-miR-212	-1.021
mmu-miR-181a	-0.883
mmu-miR-342-5p	-0.838
mmu-miR-532-5p	-0.838
mmu-miR-181b	-0.811
mmu-miR-467a	-0.801
mmu-miR-7a	-0.792
mmu-miR-151-3p	-0.753
mmu-miR-298	-0.734
mmu-miR-148a	-0.719
mmu-let-7g	-0.714
mmu-miR-106b	-0.693
mmu-miR-23a	-0.691
mmu-miR-18a	-0.664
mmu-miR-15a	-0.636
mmu-miR-21	-0.627
mmu-miR-98	-0.619
mmu-miR-872	-0.581
mmu-miR-183	-0.477
mmu-let-7f	-0.409
mmu-miR-340-5p	-0.407
mmu-miR-500	-0.407
mmu-miR-28	-0.407

mmu-miR-342-3p	-0.398
mmu-miR-128	-0.370
mmu-miR-194	-0.370
mmu-miR-146a	-0.352
mmu-miR-142-3p	-0.336
mmu-miR-103	-0.319
mmu-miR-142-5p	-0.306
mmu-miR-466l	-0.288
mmu-miR-16	-0.269
mmu-miR-31	-0.256
mmu-miR-590	-0.251
mmu-miR-147	-0.233
mmu-miR-296-5p	-0.233
mmu-miR-652	-0.224
mmu-miR-148b	-0.178
mmu-miR-467e	-0.169
mmu-let-7i	-0.132
mmu-miR-25	-0.090
mmu-miR-296-3p	-0.087
mmu-miR-466g	-0.079
mmu-miR-15b	-0.049
mmu-miR-423-5p	-0.009
mmu-miR-467c	0.033
mmu-miR-361	0.061
mmu-let-7c	0.070
mmu-miR-30d	0.070
mmu-miR-186	0.088
mmu-miR-297a	0.107
mmu-miR-9	0.125
mmu-miR-374	0.198
mmu-miR-181c	0.200
mmu-miR-92a	0.217
mmu-miR-210	0.218
mmu-miR-32	0.312
mmu-miR-425	0.317
mmu-miR-93	0.317
mmu-miR-33	0.330
mmu-miR-20a	0.354
mmu-miR-363	0.382
mmu-miR-96	0.382
mmu-miR-328	0.400
mmu-miR-141	0.400
mmu-miR-182	0.437

mmu-miR-423-3p	0.437
mmu-miR-29a	0.437
mmu-miR-191	0.437
mmu-miR-20b	0.483
mmu-miR-106a	0.556
mmu-miR-421	0.591
mmu-miR-193	0.591
mmu-miR-669c	0.657
mmu-let-7d	0.675
mmu-miR-30c	0.721
mmu-miR-378	0.777
mmu-miR-744	0.870
mmu-miR-126-3p	1.260
mmu-miR-30b	1.299
mmu-miR-375	1.345
mmu-let-7b	1.502
mmu-miR-30e	1.781
mmu-miR-200c	1.941
mmu-miR-130b	1.968
mmu-miR-720	1.977
mmu-miR-19b	2.032
mmu-miR-155	2.098
mmu-miR-125	2.135
mmu-miR-301a	2.544
mmu-miR-19a	3.224
mmu-miR-700	3.233

APPENDIX

Section 2:

Micromanaging microRNAs: Tools for modulating microRNA activity

Ectopic microRNA expression

As with many topics in molecular biology, the tools available for experimentation and manipulation of gene expression often represent an important limiting factor. MicroRNA biology is no different and involves its own unique set of limitations and considerations based on the biogenesis and mechanism of action of miRNAs. For example, when designing a miRNA expression construct for an independently transcribed miRNA, there is no start codon or stop codon to define the relevant sequence as there would be for a protein-coding gene. Without a well-defined region to insert into an expression vector, efforts have been made to determine the necessary region and features of the pri-miRNA transcript that allow processing to the active, mature form. Such work has demonstrated that inclusion of approximately 100 to 200 nucleotides on either side of the pre-miR hairpin allows effective processing to mature miRNAs in a manner indistinguishable from the endogenous miRNAs (39). This relatively simple approach is effective for efficient expression and overexpression of miRNAs in transducable, wildtype cells, but is not a practical option in cells that are lacking components of the miRNA biogenesis machinery. Of note, at least two Dicer-dependent but Drosha-DGCR8-independent microRNAs have been

identified (82) and both are expressed at relatively high levels in helper T cells (see Chapter 2). While this finding suggests the possible existence of sequence-specific or structural regulatory elements in the pri-miRNA that allow Drosha-DGCR8-independent processing, such elements have thus far not been identified. Understanding the mechanisms that underlie the processing of these miRNAs will likely have important implications for miRNA regulation and expression and may also allow improved strategies for experimental miRNA expression.

The functional expression of miRNAs in cells that lack miRNA processing machinery such as Dicer, Drosha, or DGCR8 raises both interesting challenges and opportunities. As these cells are otherwise deficient for miRNA activity, they represent a potentially useful system for replacing miRNAs one at a time to reveal functional contributions of the introduced miRNAs. The expanding use of shRNA and siRNA for gene knockdown has also accelerated the demand for various delivery methods for small RNAs. One strategy stemming from the shRNA field offers a promising means of bypassing Drosha-DGCR8 by using a polymerase III (pol III) promoter to drive transcription of a short transcript consisting of only the pre-miR hairpin sequence (142). However, it seems that even when utilizing the defined polyT termination sequence for pol III transcription to specify the length of the desired product, the resulting transcript is not always processed efficiently to the mature form (39, 143) (Figure 1A). This may be due in part to the unavoidable addition of adenine nucleotides on the 3' end of the transcript that correspond to the polyT termination signal of RNA pol III

as these extra nucleotides may interfere with subsequent Dicer processing (144). We considered this possibility when designing hairpin pre-miR expression constructs and we tried omitting the two 3' nucleotides of the complementary strand sequence that would normally constitute the 3' overhang. The idea behind this was to allow replacement of the omitted nucleotides by adenines during pol III termination to produce a Dicer-compatible substrate without an excessive 3' overhang on one end. Expression of short hairpin 'pre-miRs' did result in higher miRNA expression relative to a control vector for some miRNAs, such as miR-31 and miR-150, but the success of these expression constructs was inconsistent (Figure 1A). Additionally, the relative level of miRNA expression achieved with those constructs that "worked" was still several fold less than the baseline expression in wildtype cells (data not shown). Lastly, in some cases we observed production of the ~22mer from the complementary side of the hairpin either instead of, or in addition to, the intended mature miRNA (Figure 1B). To this extent, there is evidence that the 5' end thermodynamic stability determines which strand is ultimately loaded as the mature miRNA (145, 146). We tried to incorporate this into our design as well by including a 2-nucleotide mismatch mutation in the 3' end of the star-strand in order to destabilize the 5' end of the intended guide strand. It is unclear however if this had any effect on the strand bias of miRNA expression in our system. Finally, there is also recent crystal structure data of miRNA-loaded AGO2 that indicates an AGO2 preference for specific bases (U or A) at the 5' end of the guide strand miRNA (147). Perhaps a strategy that exploits such structural data would further direct utilization of the

intended miRNA sequence and avoid unwanted strand bias. Taken together, these findings suggest multiple layers and nuances to miRNA processing that are yet to be fully described. Additional empirical data from different expression strategies will likely be necessary to optimize short hairpin RNA expression strategies in both wildtype and microprocessor-deficient cells.

Currently, the most effective means of introducing miRNAs into DGCR8-deficient cells that we have found is the transfection of small dsRNA or “miRNA mimics”. These oligonucleotides do not necessarily contain the exact sequence of the endogenous dsRNA Dicer product on the complementary or passenger strand, but have been designed to optimize loading of the intended mature miRNA sequence. These are not without a set of caveats regarding possible indirect effects of the synthesized molecule itself or possible reproducibility issues from one source of proprietary-design mimics to the next. The use of mimics is also limited by the transient nature of their effect. As they are not being actively produced from an expression construct, dividing cells will rapidly dilute the transfected material after two to three days. This can be clearly demonstrated by the return of GFP expression from a miRNA sensor that was initially repressed following miRNA transfection (data not shown). For long term expression of individual miRNAs, as needed for most in vivo experiments, virus based expression constructs will have a significant advantage if they can be suitably optimized. Still, miRNA mimics are able to exert significant, sequence-specific functional effects on both reporter constructs and endogenous mRNA targets in a manner consistent with known functions of miRNAs. While

observations made with miRNA mimics should ideally be further validated in additional systems, they do represent a very useful tool for gaining insight into activity and potential function of individual miRNAs.

Inhibiting microRNA activity

The most common strategy for miRNA inhibition is actually quite similar to that of miRNA mimics and involves the transfection of anti-sense nucleotides that are designed to bind the mature miRNA and thus block its functional activity. Modifications to increase the stability (phosphorothioate backbone or the addition of a 3' hairpin sequence) and binding affinity (locked nucleic acid) of these molecules to their target miRNAs can further increase their effectiveness, but these molecules are still subject to turnover and dilution in rapidly dividing cells.

Another important consideration with miRNA inhibitors is their activity against a family of miRNAs when multiple miRNAs containing the same seed region are expressed. One simple approach to address this issue that has proved reasonably successful is the design of small 8-9 nucleotide inhibitors that can bind to the seed region and inhibit all members of a miRNA seed family (148). Increasing the binding affinity with LNA modifications can allow stable interaction with the target-miRNA despite the short binding sequence, but these "tiny LNAs" may still be particularly prone to off-target binding effects given the especially short length of specificity-determining sequence. Perhaps the most effective approach that we have tried is simply combining anti-sense inhibitors for multiple miRNA family members that differ at only the few positions not shared

between them. In our hands, the combination of such inhibitors proved to be the most effective way to inhibit miR-29 family members in helper T cells in vitro as evidenced by the increase in GFP expression from a GFP miR-29 sensor construct (see Chapter 2). Within this approach, hairpin antisense inhibitors did not differ from phosphorothioated LNA molecules in their efficiency of inhibition in our assays (data not shown). These experiments involved sensor analysis at only 24 or 48 hours post-transfection and further characterization of the longevity of the miRNA inhibition by these different molecules may reveal meaningful differences.

The evaluation of miRNA inhibition is, itself, not a trivial task. Simply measuring miRNA levels may not be appropriate as antisense inhibition may result in a combination of degradation and sequestration of the mature miRNA (149). Furthermore, the presence of excess modified antisense oligonucleotide may interfere with standard miRNA expression analysis (149). The use of a luciferase or GFP reporter gene with miRNA binding sites in the 3'UTR is one effective technique for measuring changes in miRNA activity. Although this type of miRNA reporter may have limited sensitivity to very small changes in miRNA activity and does require transfection or transduction of the target cell, such "miRNA sensors" can be an important tool for measuring miRNA inhibition.

The use of miRNA inhibitors in vivo is subject to additional challenges of both delivery and stability. The addition of a cholesterol modification to anti-sense inhibitors like those described above can allow passive uptake into cells, and can successfully inhibit target miRNA, but very large quantities are required for in vivo

experiments (multiple doses at ~80 mg/kg) (150). Additionally, deliberate targeting of these molecules to a specific cell type is not readily possible. Antibody decorated nanoparticles may someday be an effective option, but until then, the most effective use of miRNA inhibitors in vivo may be in the liver as this is a site of passive accumulation for intravenously delivered miRNA inhibitors (150). Ongoing experimentation in our lab with airway delivered miRNA inhibitors will hopefully offer additional insight into the types of cells that can be targeted in vivo with a different mode of delivery.

As an alternative for stable miRNA inhibition, one might be able to take advantage of the interesting relationship that has emerged between expression of miRNA target genes and activity of the miRNA itself (151, 152). Gentner et al. were able to reduce the activity of a targeted miRNA by using an expression construct with a GFP transcript containing 8 miRNA binding sites in the 3'UTR (153). When expressed at adequately high levels, this transcript could effectively act as a miRNA "sponge", binding and preventing the corresponding miRNA from acting on its endogenous targets. This of course raises intriguing possibilities about a co-regulatory relationship between miRNAs and their targets that likely exists in physiological settings as well. The use of this system as a tool, however, deserves a critical eye, for it may be difficult to determine at what point a sponge construct is expressed at sufficiently high levels to actually reduce miRNA activity against other targets. To this extent, we and others have demonstrated with both functional and reporter assays that it can be difficult to completely inhibit a highly expressed miRNA, even with high doses of antisense inhibitors. Many of the

current strategies for miRNA inhibition may ultimately be most effective against those miRNAs with relatively modest expression profiles and the continued improvement of miRNA inhibitors will no doubt have important implications for both laboratory and clinical applications. In the meantime, the utilization of conditional miRNA knockout mice will remain an invaluable tool for miRNA loss of function studies and the characterization of individual miRNAs in vitro and in vivo.

Figures

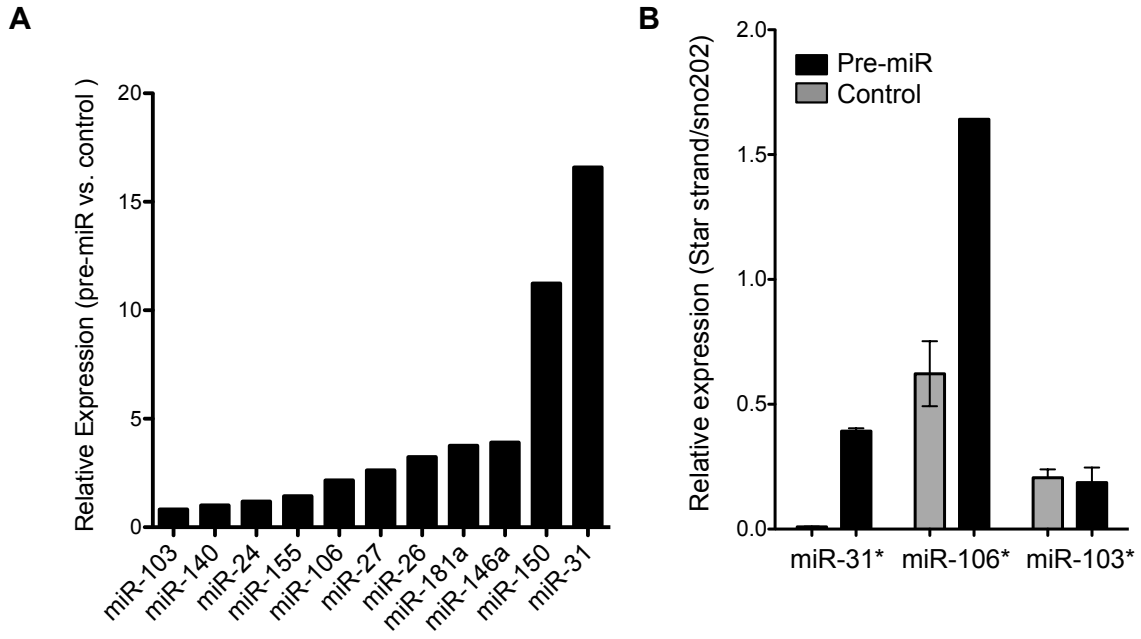

Figure 1.

MicroRNA expression using short hairpin expression constructs in DGCR8-deficient helper T cells

(A) qPCR for indicated mature miRNAs in cells transduced with a pSuper-based retroviral short-hairpin expression construct specific for indicated miRNA. Cells were sorted on Thy1.1 expression as a marker of transduced cells prior to RNA extraction for qPCR. All data are miRNA relative to sno202 RNA. This relative expression was then normalized to the relative miRNA expression in cells that were transduced with an irrelevant expression construct. Thus, the presented values represent the fold increase in miRNA expression attributed to the expression construct.

(B) RNA from cells transduced and sorted as in (A) were analyzed by qPCR for the “star-strand” of the double-stranded RNA Dicer product. Black bars are cells transduced with an expression construct for that miRNA; gray bars are cells transduced with an unmatched miRNA expression construct.

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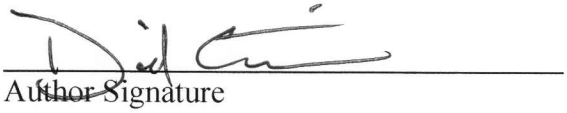

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